Electronic Nature of Neutral and Charged Two-Photon Absorbing Squaraines for Fluorescence Bioimaging Application

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1. INTRODUCTION

The synthesis and investigations of new squaraine derivatives are of great interest for a diverse range of fields, including organic photovoltaics, nonlinear optics, optical data storage, photodynamic therapy, and fluorescence bioimaging. Most of the known symmetrical squaraines are neutral compounds with electron-deficient C=O central squarainyl ring playing the role of the electron acceptor part (A), flanked by the electron-donating terminal groups (D). Together they form corresponding D–A–D-type electronic structures and can be stabilized by resonance with zwitterionic forms. Squaraine derivatives exhibit linear photophysical and nonlinear optical properties that are largely determined by the nature of the terminal substituents.

Supporting Information

ABSTRACT: The electronic properties of neutral 2,4-bis(4-bis(2-hydroxyethyl) amino-2-hydroxy-6-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)squaraine (1) and charged 2-((3-octadecylbenzothiazol-2(3H)-ylidene)methyl)-3-oxo-4-(3-(6-(pyridinium-1-yl)butyl)benzothiazol-3-ium-2-yl)methylene)cyclobut-1-enolate iodide (2) squarine derivatives were analyzed based on comprehensive linear photophysical, photochemical, nonlinear optical studies (including two-photon absorption (2PA) and femtosecond transient absorption spectroscopy measurements), and quantum chemical calculations. The steady-state absorption, fluorescence, and excitation anisotropy spectra of these new squaraines revealed the values and mutual orientations of the main transition dipoles of 1 and 2 in solvents of different polarity, while their role in specific nonlinear optical properties was shown. The degenerate 2PA spectra of 1 and 2 exhibited similar shapes, with maximum cross sections of ~300–400 GM, which were determined by the open aperture Z-scan method over a broad spectral range. The nature of the time-resolved excited-state absorption spectra of 1 and 2 was analyzed using a femtosecond transient absorption pump–probe technique and the characteristic relaxation times of 4–5 ps were revealed. Quantum chemical analyses of the electronic properties of 1 and 2 were performed using the ZINDO/S//DFT theory level, affording good agreement with experimental data. To demonstrate the potential of squaraines 1 and 2 as fluorophores for bioimaging, laser scanning fluorescence microscopy images of HeLa cells incubated with new squaraines were obtained.

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intramolecular steric hindrance and hydrogen bonding, and symmetry of the electronic distribution. Typically squaraines possess high photochemical stability. Symmetrical squaraines are characterized by relatively sharp and strong absorption bands with maximum extinction coefficient, $\varepsilon_{\text{max}} > 10^4$ M$^{-1}$ cm$^{-1}$, efficient fluorescence in the near-infrared (NIR) spectral range, and large two-photon absorption (2PA) cross-sections, $\sigma_{\text{2PA}}$ up to $10^4$ GM. Large 2PA cross-sections are a result of the large $\varepsilon_{\text{max}}$ combined with double resonance. These properties are promising for applications in two-photon-induced fluorescence microscopy (2PFM) bioimaging and it is a subject of active interest. Unsymmetrical squaraines typically exhibit much broader and less intense absorption bands and can be used for the development of organic solar cell technologies.

Charged squaraine molecules are scarcely addressed in the scientific literature and are mainly characterized by specifically designed substituents with an uncompensated charge that does not noticeably change the electronic distribution of the primary $\pi$-electron system. These charged substituents may have a variety of desired effects, such as increased bonding sensitivity for specific biomolecules, strongly modified aggregation phenomena, and increased sensor’s sensitivity.

In this work, we present the synthesis and comprehensive linear photophysical and nonlinear optical characterization of neutral (1) and charged (2) squarine derivatives with potential for bioimaging applications. The nature of the electronic structures, 2PA efficiency, and fast relaxations in the excited state of 1 and 2 were revealed using a 1 kHz femtosecond laser system along with the quantum-chemical analysis at the ZINDO/S//DFT levels of theory. Fluorescence bioimaging of HeLa cells was accomplished under one-photon excitation for 1, and one- as well as two-photon excitations for 2 to demonstrate their potential ability as probes in laser scanning fluorescence microscopy.

2. EXPERIMENTAL SECTION

2.1. Design and Synthesis of 1 and 2. Squaraines 1 and 2 (Figure 1) were obtained via the condensation of electron-rich aromatic precursors with squaric acid in a one-pot reaction using a Dean–Stark water separator. The chemical structure and purity of the related compounds were confirmed by $^1$H NMR, $^{13}$C NMR, and high-resolution mass spectroscopy. An overview of the synthetic procedures and molecular characterization details are illustrated in the Supporting Information. Briefly, based on nucleophilic substitution with 1-(2-bromoethoxy)-2-(2-methoxyethoxy)ethane under basic conditions, 5-(bis[2-hydroxyethyl]amino) benzene-1,3-diol was converted into compound A (see Scheme S1). For the unsymmetrical squarine 2, two different activated heterocyclic compounds, B and C (see Scheme S2), were synthesized based on the general reactivity of 2-methylbenzothiazole with alkyl iodide derivatives under heating in a microwave reactor. The two different activated heterocyclic compounds were then subjected to a condensation reaction in a 1:1 ratio, resulting in a 10% yield of 2 after purification. Significantly, when the last step is performed in this way, the separation of the semisquaraine can be avoided.

2.2. Linear Photophysical and Photochemical Measurements. Linear spectroscopic characterization of 1 and 2 was performed at room temperature in air-saturated spectroscopic grade tetrahydrofuran (THF), dichloromethane (DCM), dimethyl sulfoxide (DMSO), and acetone (ACN). All solvents were purchased from commercial sources and used without further purification. The steady-state one-photon absorption (1PA) spectra of 1 and 2 were measured in 1 cm path length quartz cuvettes using a Varian Cary-500 spectrophotometer and solute concentrations $C \approx (6–8) \times 10^{-6}$ M. The steady-state and time-resolved fluorescence parameters, such as corrected fluorescence, excitation, and excitation anisotropy spectra, fluorescence quantum yields, $\Phi_{\text{fl}}$, and corresponding lifetimes, $\tau_{\text{fl}}$ of 1 and 2 were obtained for dilute solutions ($C < 10^{-6}$ M) using a FLS980 spectrofluorimeter (Edinburgh Instruments Ltd.) in standard 1 cm path length spectrophotometric quartz cuvettes. The values of $\Phi_{\text{fl}}$ were determined by a relative method with cresyl violet in CH$_3$OH as a reference. The steady-state excitation anisotropy spectra of 1 and 2, $r(\lambda) = r_0(\lambda)/(1 + r_0/\theta)$ (where $r_0(\lambda)$, $\theta = \eta V/kT$, $\eta$, $V$, $k$, and $T$ are the fundamental anisotropy spectrum, rotational correlation time, solvent viscosity, effective rotational volume of the molecule, Boltzmann’s constant, and absolute temperature, respectively), were measured in viscous polytetrahydrofuran (pTHF) and glycerol at room temperature. Under these experimental conditions $\theta \gg r_0$ (i.e., depolarization processes related to molecular rotational motion are negligible) and the fundamental anisotropy spectrum can be approximated by $r_0(\lambda) \approx r(\lambda)$. The level of photochemical stability of 1 and 2 were characterized by the photodecomposition quantum yield, $\Phi_{\text{ph}}$, equal to the number of decomposed molecules divided by the number of absorbed photons, that can be determined by the expression:

$$\Phi_{\text{ph}} = \frac{\int_0^\infty \frac{[D(\lambda, 0) - D(\lambda, t_u)] \cdot N_{\text{ch}}}{\varepsilon(\lambda) \cdot 10^3 \cdot \int_0^\infty I_0(\lambda) \cdot [1 - 10^{-D(\lambda, t_u)}]} \cdot d\lambda \cdot dt}$$

where $D(\lambda, 0)$, $D(\lambda, t_u)$, $N_{\text{ch}}$, $\varepsilon(\lambda)$, $\lambda$ and $t_u$ are the values of initial and final absorbance of the solution, Avogadro’s number, extinction coefficient (M$^{-1}$ cm$^{-1}$), excitation wavelength (nm), and irradiation time ($s$), respectively; $I_0(\lambda)$ is the spectral distribution of the excitation irradiance. Equation 1 corresponds to an absorption method, comprehensively described in ref S8, and the values of $\Phi_{\text{ph}}$ were determined under continuous wave diode laser irradiation of 1 and 2 into the main absorption band ($\lambda = 650$ nm, average beam irradiance $\approx 50$ mW/cm$^2$).
2.3. 2PA Spectra and Femtosecond Transient Absorption Pump–Probe Measurements. The degenerate 2PA spectra of 1 and 2 were obtained in THF (1) and ACN (2) at room temperature in 1 mm path length quartz cuvettes with molecular concentrations $C \approx 10^{-3}$ M, using an open aperture Z-scan method.\(^5\)\(^9\) Transient absorption pump–probe measurements\(^2\)\(^8\)\(^9\)\(^{60}\) were performed in DCM (1) and ACN (2) at room temperature in a 1 mm quartz flow cell to minimize thermo-optical distortion and photodecomposition processes. Experimental details of the femtosecond laser system and process employed were comprehensively described previously.\(^5\)\(^6\)\(^9\) In brief, the exit laser beam of a Ti:sapphire regenerative amplifier Legend Duo+ (Coherent, Inc.) at $\lambda = 800$ nm, with 1 kHz repetition rate, pulse duration, $t_p \approx 40 \text{ fs}$, and pulse energy, $E_p \approx 12 \text{ mJ}$, was split in two separate channels for Z-scan and pump–probe experimental setups. The first beam pumped an optical parametric amplifier HE-TOPAS (Light Conversion, Inc., tuning range 1500–2800 nm), and the output beam was frequency-doubled with a 1 mm BBO crystal and filtered by spike filters with transmission bands of $\approx 10$ nm (fwhm). The resulting laser channel was tuned in the range $\sim 750$–1400 nm with $t_p \approx 100 \text{ fs}$, pulse energy, $E_p \leq 10 \mu J$, and used for Z-scan measurements. The second part of the split output of the Legend Duo+ at 800 nm was attenuated and focused in a standard quartz cell with water (1 cm path length), producing a white light continuum as a probe beam for pump–probe measurements. The pump beam for this setup was taken from the Z-scan channel tuned to 650 nm. The transmitted probe signal was detected with an Acton SpectraPro 2560i spectrograph and PI-MAX2 ICCD camera (all Princeton Instruments, Inc.). The time resolution of the pump–probe setup was estimated to be $\sim 200 \text{ fs}$.

2.4. Computational Details. All quantum-chemical calculations were performed with Gaussian 2009, Rev.A02.\(^6\)\(^2\) The density functional theory (DFT) level B3LYP/6-31G* was used for geometry optimization, while a semiempirical ZINDO/S method\(^6\)\(^3\) was adopted to describe the electronically excited states. For compound 2, several ion pair configurations were used as starting points. They all converged to two configurations with iodide anion in the molecular plane. Out of those, the lowest energy one was used for the spectral predictions (the iodide was replaced with fluoride). The 2PA spectra of 1 and 2 were calculated with an in-house script\(^6\)\(^4\)\(^6\)\(^5\) implementing the sum over states (SOS) formalism\(^6\)\(^6\) with six excited states and empirical broadening parameter, $\Gamma \approx 0.1$ eV. Following the previously proposed strategy,\(^5\)\(^1\) the empirical parameters (common for both chromophores and equal to $p_p = 1.266$ and $p_d = 0.530$) were adjusted in modified Gaussian code to fit the longest observed wavelengths in the 1PA spectra.

2.5. Cell Culture and Incubation. To estimate the cytotoxicity of compound squaraine 1 and 2, HeLa cells were cultured in Minimum Essential Medium supplemented with 10% fetal bovine serum and 1% penicillin, 1% streptomycin at 37 °C, in a 95% humidified atmosphere containing 5% CO$_2$. Cells were seeded in 96-well plates (Corning, USA) at a concentration of $5 \times 10^3$ cells/well for 48 h. Next, HeLa 116 cells were incubated with different concentrations of compound 1 or 2 for an additional 22 h. After that, $20 \mu L$ of Cell Titer 96 Aqueous One solution reagent was added into each well followed by incubation for 2 h additionally. The respective absorbance values were measured on a Tecan Infinite M200 PR plate reader spectrometer at 490 nm to determine the relative amount of formazan produced. Cell viabilities were calculated based on the following eq 2:

$$\text{Cell viability (\%)} = \frac{\text{Abs}_{490nm}^S - \text{Abs}_{490nm}^D}{\text{Abs}_{490nm}^C - \text{Abs}_{490nm}^{D2}} \times 100\%$$

where $\text{Abs}_{490nm}^S$ is the absorbance of the cells incubated with different concentrations of the dye solutions, $\text{Abs}_{490nm}^D$ is the absorbance of cell-free well containing only dye at the investigated concentration, $\text{Abs}_{490nm}^C$ is the absorbance of the cells only incubated in medium, and $\text{Abs}_{490nm}^{D2}$ is the absorbance of the cell-free well.

2.6. One- and Two-Photon Fluorescence Microscopy Imaging. HeLa cells were placed onto poly-d-lysine coated coverslips in 24-well plated at the density of $5 \times 10^3$ cells/well and incubated for 48 h. The investigated squaraine 1 solution was diluted to the concentration of 40 $\mu$M, and squaraine 2 solution was diluted to the concentration of 10 $\mu$M, respectively. After 2 h incubation, the solutions were extracted and the
Fluorescence cell images were obtained on an inverted fluorescence microscopy system equipped with a Q-Imaging-cooled CCD and a 100 W mercury lamp. The TexRed microscope (Olympus IX71) equipped with a Q-Imaging-filter cube (Ex: 562/40 nm; DM: 593 nm; Em: 624/40 nm) was used to match the excitation wavelength of squaraine 1 or 2 and to capture most of its emission profile. A customized filter cube (Ex: 377/50 nm; DM: 420 nm; Em: 460/40 nm) was used for the nucleic acid imaging.

Two-photon fluorescence images were recorded with a Bruker Ultima fluorescence microscopy system equipped with a Coherent Mira 900F laser source (76 MHz, mode-locked, 200 fs pulse width). The fs laser excitation was tuned to 820 nm in order to achieve the peak of fluorescence.

coverslipped cells were washed abundantly with phosphate-buffered saline (PBS) buffer (2×). After incubation with medium containing 1 µg/mL Hoechst for nucleic acid staining, cells were fixed with 3.7% formaldehyde solution in PBS buffer at room temperature for 10 min. The fixing agent was extracted and washed (2×) with PBS. A fresh solution of NaBH4 (1.0 mg/mL) was used to treat the fixed cells for 10 min (2×) to reduce the autofluorescence. The coverslipped cells were then washed with PBS buffer (2×), deionized water (1×), and mounted on microscope slides using an antifade mounting medium (Prolong Gold) for imaging.

Fluorescence cell images were obtained on an inverted microscope (Olympus IX71) equipped with a Q-Imaging-cooled CCD and a 100 W mercury lamp. The TexRed filter cube (Ex: 562/40 nm; DM: 593 nm; Em: 624/40 nm) was used to match the excitation wavelength of squaraine 1 or 2 and to capture most of its emission profile. A customized filter cube (Ex: 377/50 nm; DM: 420 nm; Em: 460/40 nm) was used for the nucleic acid imaging.

Two-photon fluorescence images were recorded with a Bruker Ultima fluorescence microscopy system equipped with a Coherent Mira 900F laser source (76 MHz, mode-locked, 200 fs pulse width). The fs laser excitation was tuned to 820 nm in order to achieve the peak of fluorescence.

### 3. RESULTS AND DISCUSSION

#### 3.1. Linear Photophysical Properties and Photochemical Stability of 1 and 2

The steady-state 1PA spectra of new symmetrical 1 and unsymmetrical 2 squaraine derivatives exhibited similar sharp and narrow main absorption bands at ~640–680 nm (Figure 2, curve 1) with maximum extinction coefficient of $e_{\text{max}} \approx (1.5–1.8) \times 10^4$ M$^{-1}$ cm$^{-1}$ (see Table 1), which is typical for symmetrical squaraine structures with absorption maxima in the 600–700 nm spectral range.\(^{24,30,33}\) This means that the symmetry of the main π-electron system in 2 is not noticeably affected by the charged substituent in the molecular structure, while a weak solvatochromic behavior of the absorption spectra was observed (Table 1). Aggregation effects of 1 and 2 were negligible in all employed solvents with solute concentrations $C \leq 10^{-3}$ M. The steady-state fluorescence spectra of 1 and 2 (Figure 2, curves 2) exhibited relatively small Stokes shifts, typical for symmetrical squaraines,\(^{20,53,57}\) and were independent of excitation wavelength, $\lambda_{\text{exc}}$, over the entire absorption range. The values of the fluorescence quantum yields, $\Phi_f$, were strongly dependent on solvent polarity (see Table 1) without any dependence on $\lambda_{\text{exc}}$, and no violations of Kasha’s rule\(^{56}\) were observed. Taking into account similar shapes and spectral positions of the absorption and emission spectra of the new squaraines in different solvents, nearly equal extinction coefficients, and strong dependence of $\Phi_f$ on solvent polarity, it can be assumed that solvation processes mainly affect...
the radiationless intramolecular transitions in 1 and 2 without noticeable influence on the radiative transition dipoles. Both compounds exhibited single-exponential fluorescence decay in all solvents with characteristic lifetimes, $\tau_0$, in the range 0.8–1.7 ns. The values of $\tau_0$ can be calculated based on the Strickler–Berg approach$^{67}$ and corresponding lifetimes ($\tau_{ph}^{ab}$) are presented in Table 1. According to these data, experimental and calculated values are in acceptable agreement with each other, as evidenced by good mirror symmetry between absorption and fluorescence spectra and small changes in the optimized molecular geometry of 1 and 2 under electronic excitation.$^{68}$

The excitation anisotropy spectra of 1 and 2, $r(\lambda) = r_0(\lambda)/(1 + r_0(\lambda)/\theta)$ (see Section 2.2), were measured in solvents of different viscosity (Figure 3), and the resulting data along with linear photophysical parameters are presented in Table 1, revealing the nature of the main absorption bands, relative orientations of the transition dipoles, rotational correlation times, $\theta$, and effective rotational volumes, $V$. The nearly constant value of anisotropy in the main 1PA band (i.e., $\lambda \approx 600–700 \text{ nm}$) is evidence of a single electronic transition $S_0 \rightarrow S_1$ of 1 and 2 in this spectral range ($S_0$ and $S_1$ are the ground and first excited electronic state, respectively).$^{69}$ The values of fundamental anisotropies, $r_0(\lambda)$, were measured in viscous solvents (i.e., pTHF and glycerol at room temperature), where rotational depolarization processes are negligible and $r_0(\lambda) \approx r(\lambda)$. The maximum value of $r_0(\lambda)$ is determined by the angle, $\alpha$, between the absorption ($S_0 \rightarrow S_1$) and emission ($S_1 \rightarrow S_0$) transition dipoles and can be expressed as $r_0 = (3 \cos^2 \alpha - 1)/5$.$^{56}$ Relatively high values of $r_0 \approx 0.35–0.37$ in the main absorption bands are supportive of a close to parallel orientation of the corresponding absorption and emission transition dipoles of 1 and 2 (i.e., $\alpha \leq 13–17^\circ$). In this case, it is possible to estimate relative orientations of the absorption dipoles $S_0 \rightarrow S_1$ ($S_0$ are higher excited electronic states).$^{70}$ Dramatic changes in the excitation anisotropy spectra of 1 and 2 in the short wavelength range ($\lambda_{\text{ex}} < 550 \text{ nm}$) reflect the sufficiently different orientation of the $S_0 \rightarrow S_1$ transition dipoles relative to $S_0 \rightarrow S_1$ ($25^\circ < \alpha < 50^\circ$). The observed maximum (for 1) and minimum (for 2) at $\approx 350 \text{ nm}$ are in good agreement with the spectral position of the corresponding weak short wavelength absorption bands (see insets in Figure 3), and indicate the relatively small ($\alpha \approx 20^\circ$) and large ($\alpha \approx 50^\circ$) angles between $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$ transition dipoles for 1 and 2, respectively.

As seen in the data for both compounds 1 and 2 in Table 1, the values of the rotational correlation time, $\theta$, exhibit nearly linear dependence on solvent viscosity, $\eta$, and a correspondingly weak dependence of the effective rotational volume, $V$, on solvent properties in accordance with $\theta = \eta V/kT$. This suggests that for each squaraine dye, nearly the same volumetric shapes of the solvated molecular structure are formed in different solvents.

The photostability of 1 and 2 were characterized by an absorption method detailed previously,$^{58}$ and corresponding photodecomposition quantum yields, $\Phi_{ph}$, were determined using eq 1 with data presented in Table 1. According to these data, neutral squaraine 1 exhibited sufficiently high photostability, $\Phi_{ph} \approx 10^{-6}$, comparable to the corresponding values of laser dyes.$^{71,72}$ In contrast, the photodecomposition processes for charged squaraine 2 were much more efficient in all employed solvents ($\Phi_{ph} \approx 10^{-4}$); however, this level of stability is still acceptable for its practical application in bioimaging.$^{73}$

3.2. 2PA and Femtosecond Transient Absorption Spectroscopy of 1 and 2. The investigation of the 2PA properties of squaraines 1 and 2 were performed over a broad spectral range (740–1400 nm) using an open aperture Z-scan technique$^{59}$ with the corresponding degenerate spectra presented in Figure 4 (curves 1, 2). We use different solvents because of different solubility and higher concentration which is important in Z-scan measurements. We know that solvatochromic effects are not strong for the investigated squaraines, and we can expect that 2PA spectra do not exhibit a strong dependence on solvent polarity. Accordingly, the 2PA spectra exhibited two well-defined bands at $\approx 600$ and $\approx 380 \text{ nm}$ for 1, and $\approx 620$ and $\approx 500 \text{ nm}$ for 2. The observed long wavelength, weak 2PA maxima ($\delta_{2PA} \approx 100 \text{ GM}$) are typical for centrosymmetrical squaraine derivatives and can be assigned to the spectral position of the asymmetric vibrational modes of the main linear absorption band.$^{33,74}$ It also means, that the shape of the main $\pi$-electron system of 2 is sufficiently close to the centrosymmetrical structure, and the charged, nonconjugated substituent does not play a substantial role in the corresponding nonlinear optical properties.

The values of 2PA cross-sections noticeably increase in the short wavelength region up to $\delta_{2PA} \approx 420 \text{ GM}$ and $\delta_{2PA} \approx 260 \text{ GM}$ for 1 and 2, respectively. These 2PA maxima are related to two-photon allowed $S_0 \rightarrow S_2$ electronic transitions, as can be concluded from the steady-state excitation anisotropy spectra (Figure 3, curves 1) and very weak 1PA efficiency in this spectral range. It should be mentioned that the $\delta_{2PA}$ for 1 and 2 are smaller than previously reported values for symmetrical squaraine derivatives.$^{12,20}$ Based on a simple three-level model for 2PA processes,$^{75}$ this can be explained by the relatively small extinction coefficients, $\epsilon(\lambda_{\text{max}})$, and corresponding reduced values of the related transition dipoles for $S_0 \rightarrow S_1$ transitions, $\mu_{0i} \approx 0.996 \sqrt{\epsilon(\nu, \lambda_{\text{max}}) \delta V} (\mu_0 i$ in deby, $\nu$ in cm$^{-1}$, $\lambda$ in cm)$^{76}$. The potential of 1 and 2 for application in laser scanning fluorescence microscopy techniques can be estimated using a “figure of merit,” $F_M = \delta_{2PA} \Phi_{ph}/\Phi_{ph} \approx 10^6$ to $10^{10}$, which is comparable with known two-photon fluorescent molecular probes for bioimaging.$^{35,77}$

Excited-state absorption (ESA) and fast relaxation processes in the ground and excited states of 1 and 2 were investigated using a femtosecond transient absorption pump–probe technique under excitation in the main long wavelength 1PA band ($\lambda_{\text{ex}} = 650 \text{ nm}$). Temporal dependences of the induced optical density, $\Delta D$, on the delay between pump and probe pulses, $\tau_{\text{dB}}$, were obtained over a broad spectral range, and corresponding dependences, $\Delta D(\tau_{\text{dB}})$, along with the transient absorption spectra, are presented in Figures 5 and 6. The main...
ESA bands were observed in the short wavelength region at ∼450–530 nm (for 1) and ∼500–570 nm (for 2) that are typical for symmetrical squaraines with $\lambda_{ab}^{\text{max}}$ at ∼600–700 nm. It should be mentioned that gain (i.e., optical amplification via stimulated emission) is not possible in the spectral range $\lambda < 600$ nm, and saturable absorption (SA) is negligible because of a weak linear absorption at $\lambda < 570$ nm for both molecules. Observed negative changes in the optical densities (i.e., $\Delta D < 0$) in the spectral range of 580–680 nm for 1 and 600–700 nm for 2 are related to SA processes and some possible inputs of gain in the corresponding fluorescence contours (Figure 6c,d, curves (3)). Negative values of $\Delta D$ for $\lambda \geq 680$ nm (for 1) and $\lambda \geq 700$ nm (for 2) are mainly determined by the gain phenomena. No superluminescence or lasing was observed for these squaraines, presumably because of their relatively low-fluorescence quantum yields (see Table 1). Fast but weak relaxation processes were observed for 1 in DCM with a characteristic time of 4–5 ps (Figure 6a) that can be related to solvent reorganization phenomena in low-viscosity solvents after $S_0 \rightarrow S_1$ excitation. By contrast, the charged squaraine 2 did not exhibit any fast relaxation in the $S_1$ excited electronic state, and after excitation the value of $\Delta D$ relaxed to zero in accordance with its nanosecond fluorescence lifetime, $\tau_{fl}$.

3.3. Electronic Structure Analysis. The electronic transitions of 1 and 2 were described based on a ZINDO/S//DFT theoretical approach (see Section 2.4). The optimized molecular geometry, essential molecular orbitals, and calculated parameters of the lowest singlet excited electronic states of 1 and 2 are presented in Figures 7 and 8 and Table 2, respectively. Optimized molecular geometries of 1 and 2 were obtained for

![Figure 5](image-url) Contour plots of the induced optical density $\Delta D$ with $\lambda_w = 650$ nm as a function of probe wavelength, $\lambda_{pr}$, and $\tau_{D}$ for 1 in DCM (a) 0–12 ps, (b) 0–80 ps, and 2 in ACN (c) 0–14 ps, (d) 0–80 ps, obtained with femto- (a,c) and picosecond (b,d) resolution.

![Figure 6](image-url) Transient absorption dependences $\Delta D(\tau_{D})$ with $\lambda_w = 650$ nm for 1 in DCM (a) $\lambda_{pr} = 500$ nm (1), 680 nm (2), and for 2 in ACN (b) $\lambda_w = 550$ nm (1), 700 nm (2). Transient absorption spectra of 1 in DCM (c) and 2 in ACN (d) with $\lambda_w = 650$ nm for $\tau_D \approx 5$ ps (1), normalized steady-state absorption (2) and fluorescence (3).
are well-separated from others (Figure 9) and can be assigned to corresponding single electronic S1 states in the 550 structures. Calculated long wavelength electronic bands of without taking into account vibrational modes of the squaraine were acceptably described by the theoretical approach employed calculated electronic parameters of unsymmetrical electronic states and presented in Figure 9. The obtained 2PA spectra with maximum 2PA (see Figure 7b and 8b, the structure of the conjugated π-electron system of the charged compound 2 is close to centro-symmetric, which is in agreement with the experimentally observed spectral shape and position of the main long wavelength 1PA contour.

Figures 7b and 8b, the structure of the conjugated π-electron system of the charged compound 2 is close to centro-symmetric, which is in agreement with the experimentally observed spectral shape and position of the main long wavelength 1PA contour.

The calculated parameters of the lowest excited states and oscillator strengths of the corresponding electronic transitions (see Table 2) revealed a good correlation with experimental data.

It should be mentioned that the linear 1PA spectra of 1 and 2 were acceptably described by the theoretical approach employed without taking into account vibrational modes of the squaraine structures. Calculated long wavelength electronic bands of 1 and 2 are well-separated from others (Figure 9) and can be assigned to corresponding single electronic S1 states in the 550–700 nm spectral range (Table 2). This is experimentally confirmed by the constant values of anisotropy in the range of the main 1PA bands (see Figure 3, curves 1). The degenerate 2PA spectra of 1 and 2 were calculated using the SOS approach with 6 electronic states and presented in Figure 9. The obtained 2PA spectra with maximum 2PA ≈ 350–450 GM are in acceptable agreement with the experimental ones (Figure 4, curves 1, 2), except for the blue shift of the calculated 2PA maximum for 2 and longer wavelength, weak 2PA bands in the spectral range of two-photon forbidden transitions (i.e., one-photon allowed 1PA bands). These transitions can be experimentally observed for centrosymmetric molecules because of the vibronic interaction with the nonsymmetric vibrational mode. It is interesting to mention that in contrast to strictly centrosymmetric squaraine 1, calculated electronic parameters of unsymmetrical 2 reveal nonzero 2PA max in the main long wavelength 1PA band, while all its linear spectral properties strongly suggest a nearly centrosymmetric structure for 2.

3.4. Cell Viability Measurement. To ascertain the potential utility of fluorescent probe squaraine 1 and 2 for fluorescence microscopy, their viability was evaluated using HeLa cells via the MTS assay (Figure 10). After 24 h incubation with various concentrations, HeLa cells exhibited relative high viability with concentration up to 40 μM for squaraine 1, and 10 μM for squaraine 2. The data indicated that both squaraine 1 and 2 have low cytotoxicity over a concentration range of 0.5–40 μM and 0.625–10 μM, respectively, appropriate for cell imaging.

3.5. One- and Two-Photon Cell Bioimaging with New Squaraines. On the basis of cell viability test results, 30 μM squaraine 1 and 10 μM squaraine 2 in solution were used for fluorescence cell imaging for the HeLa cell line. As presented in Figure 11, cells incubated with compound 1 exhibited bright fluorescence in the cytoplasm area. In comparison, dye accumulation surrounding the nucleus area was observed for cells incubated with squaraine 2 (Figure 12). Fluorescence microscopy imaging results indicate that both compound 1 and 2 have great potential in the application of in vivo bioimaging.

To further investigate the potential use of compound 2 in imaging studies, two-photon fluorescence microscopy in vitro
cell imaging was conducted. As presented in Figure 13, HeLa cells incubated with 10 μm compound 2 exhibited bright fluorescence in the perinuclear area, consistency with the one-photon image result. The excitation wavelength employed in 2PFM imaging experiments was 820 nm, which is in the NIR window, facilitating deeper penetration compared with typical one-photon excitation in the visible range. This result and low cytotoxicity demonstrated that the squaraine 2 is a promising candidate for in vivo imaging studies.

4. CONCLUSIONS

Linear photophysical, photochemical, and nonlinear optical properties of new squaraines 1 and 2 were comprehensively investigated in liquid media at room temperature. The neutral centrosymmetric 1 and unsymmetric derivative with charged nonconjugated substituent 2 exhibited very close linear spectral parameters, including relatively narrow and sharp absorption and fluorescence peaks with small Stokes shifts and only weak dependence on solvent polarity. The nonconjugated charged substituent in 2 did not noticeably change the symmetry of the main π-electron system, but dramatically decreased its photo-stability. The steady-state excitation anisotropy spectra of 1 and 2 revealed only one electronic transition S₀ → S₁ in the main long wavelength 1PA band and its nearly parallel orientation to the corresponding emission transition dipole S₁ → S₀ that is similar to symmetrical squaraine derivatives. The degenerate 2PA spectra of new squaraines with cross-sections δ₂PA max ≈ 260–420 GM at the maxima were obtained by an open aperture Z-scan method over a broad spectral range using femtosecond excitation, revealing two well-defined 2PA bands for each squaraine. The most intense ESA spectra of 1 and 2 were found in the short wavelength range, 450–550 nm, while fast relaxation processes in the excited state were observed for neutral squaraine 1 with a characteristic time of 4–5 ps. Quantum chemical calculations of the main electronic parameters of these two squaraine derivatives, including 1PA and 2PA spectra, were performed with the ZINDO/S//DFT level of theory. We found acceptable agreement with the experimental data, except for the spectral position of the main 2PA peak for 2 and weak 2PA.
intensity in the main one-photon allowed absorption band of 1 and 2. The one-photon fluorescence imaging results demonstrated the potential ability of squaraine 1 and 2 that can be applied in the bioimaging study. In particular, HeLa cells incubated with compound 2 exhibited bright fluorescence upon two-photon excitation which indicates that this compound is also a promising candidate for two-photon fluorescence imaging studies.

The linear photophysical and nonlinear optical properties of the new squaraines, acceptable photostability ($F_{M} > 10^6$), and preliminary two-photon fluorescence bioimaging with 1 and 2 suggest their potential for use as fluorescence contrast agents in laser scanning fluorescence microscopy imaging applications.

**ASSOCIATED CONTENT**

**Supporting Information**
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00718.

Description of the employed materials and methods, synthetic route of new squaraines, and corresponding NMR and mass spectra (PDF)

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