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Dipolar Dyes

Dipolar Dyes with a Pyrrolo[2,3-*b*]quinoxaline Skeleton Containing a Cyano Group and a Bridged Tertiary Amino Group: Synthesis, Solvatofluorochromism, and Bioimaging

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Abstract: Two strongly polarized dipolar chromophores possessing a cyclic tertiary amino group at one terminus of the molecule and a CN group at the opposite terminus were designed and synthesized. Their rigid skeleton contains the rarely studied pyrrolo[2,3-*b*]quinoxaline ring system. The photophysical properties of these regioisomeric dyes were different owing to differing π conjugation between the CN group and the electron-donor moiety. These dipolar molecules showed very intense emission, strong solvatofluorochromism, and sufficient two-photon brightness for bioimaging. One of these regioisomeric dyes, namely, 11-carbonitrile-2,3,4,5,6,7-hexahydro-1*H*-3a,8,13,13b-tetraazabenzo[*b*]cyclohepta[1,2,3-*jk*]fluorene, was successfully utilized in two-photon imaging of mouse organ tissues and showed distinct tissue morphology with high resolution.

Introduction

The design of new organic chromophores for two-photon excited fluorescence microscopy (TPFM) of biological objects has attracted significant attention,[1,2] as this technique is an efficient means to study biological processes in live tissues and offers many advantages over traditional imaging methods.[3] As larger quadrupolar and octupolar, nonlinear absorbing dyes have difficulty in crossing cell membranes, small molecules tend to be more promising two-photon absorbing chromophores for fluorescence imaging purposes.[4–8] Push–pull chromophores are the prevailing variety of smaller fluorophores, as they have been known and studied for a long time[9] and have still been a subject of active investigation in recent decades.[10] Derivatives of both aromatic hydrocarbons[11] (especially 2,6-disubstituted naphthalene, i.e., acedan)[9a,11a,12] and heterocyclic systems[13] have been intensively studied. In this last case, special attention has been given to coumarins owing to their large dipole moments and high photostability combined with straightforward synthesis.[14] New trends include studies of π-expanded coumarins[15] and redshifting the emission bands while combating the decrease in the fluorescence quantum yield in polar solvents[16] by introducing superstrong acceptors[17a–d] or more powerful electron-donating groups[17e–h] (including bridged, cyclic tertiary amines).[18] Bridged and cyclic amine donors in dipolar dyes restrict internal rotations, which thereby stops nonradiative excited-state decay channels, but they require multistep syntheses. A solution to the synthetic difficulty is to utilize the bidentate nucleophilic character of 1,8-diazabicyclo[5.4.0]undec-7-en (DBU), which upon reacting with electrophiles gives rise to compounds possessing cyclic tertiary amino groups directly attached to aromatic system.[19]

The goal of this project was to further strengthen the dipolar interactions in 2-amino-1,4-diazaindole derivatives, which can be synthesized from 2,3-dichloroquinoxalines and DBU in one step according to a procedure reported by us.[20] We reasoned that placing a cyano group at the opposite side of the planar heterocycle from a tertiary amino group would further polarize the whole system and lead to desirable optical response. In combination with the small size of the molecules, this approach should open an avenue to imaging studies of cells and tissues. Herein, we reveal the synthetic and photophysical details of our study as well as two-photon excited fluorescence microscopy experiments.

Results and Discussion

2,3-Dichloro-6-cyanoquinoxaline (**2**) was chosen as the precursor for the designed dipolar heterocyclic system. This com-

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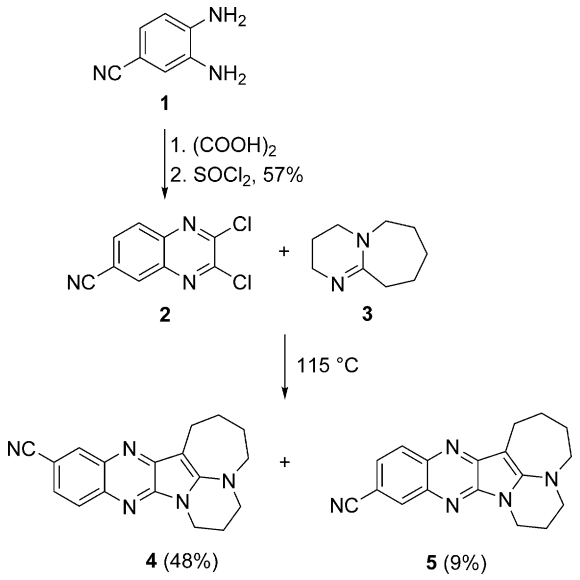


Scheme 1. Synthesis of compounds **4** and **5**.

pound was obtained in a two-step reaction according to a previously described method[21] in 57% yield (Scheme 1).

The reaction was conducted by following the general experimental procedure[20] under previously optimized conditions (115 °C, 20 min). According to our expectations, 2,3-dichloro-6-cyanoquinoxaline (**2**) was treated with DBU to provide a mixture of two regioisomers (**4** as the major product at the expense of compound **5**) (Scheme 1).

Despite very similar retardation factors, the mixture was successfully separated by column chromatography (**4**/**5** = 6:1). The nonstatistical ratio of the reaction products can be rationalized by the electronic structure of quinoxaline **2**, which consists of two C–Cl bonds with different reactivities. The cyano group at the 6-position exerts a mesomeric effect that strongly affects the 2-position towards nucleophilic attack. The fact that dye **4** formed preferentially suggests that contrary to what we hypothesized earlier,[20] initial attack takes place on the nitrogen atom of DBU (and not on the β-carbon atom).

Spectroscopic analyses confirmed the identity of regioisomeric products **4** and **5** but did not allow for structure assignment. Consequently, X-ray analysis of a more polar product was performed, which revealed its structure as **4** (Figure 1). In analogy to previously studied pyrrolo[2,3-*b*]quinoxalines,[20] the molecule is essentially planar. The well-known character of this

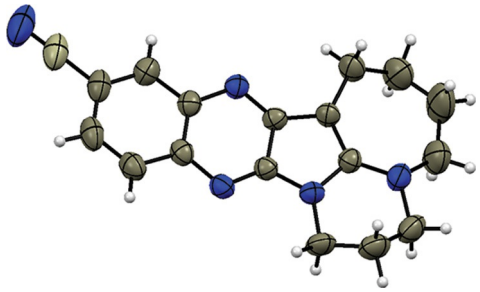


Figure 1. X-ray structure of compound **4** (CCDC 1456392).

reaction[20] and fully consistent analytical data (^{1}H NMR and ^{13}C NMR spectroscopy, HRMS), in conjunction with X-ray assignment for compound **4**, made it possible to unambiguously assign the structure of the second product as **5**.

The absorption spectra of both regioisomers in cyclohexane, toluene, and methanol are shown in Figure 2. The lowest energy absorption maxima (λ_{abs}) and extinction coefficients (ε) are collected in Table 1. Clearly, the λ_{abs} of both isomers showed a bathochromic shift from approximately $\lambda = 426$–428 nm (i.e., violet photons) in nonpolar solvents to $\lambda = 443$–449 nm in polar solvents.

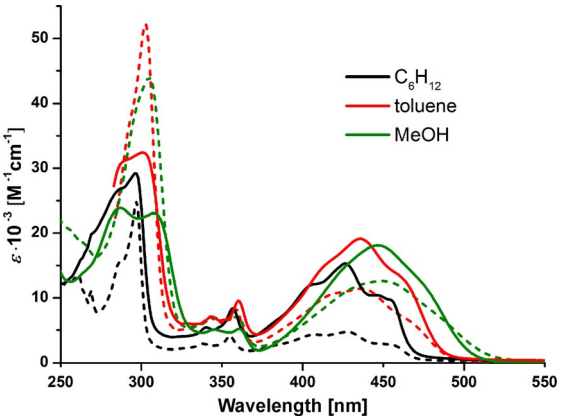


Figure 2. Absorption spectra of isomers **4** (dotted line) and **5** (solid line) in cyclohexane, toluene, and methanol at room temperature.

The fluorescence spectra of isomers **4** and **5** are shown in Figure 3. Both compounds strongly emit green-yellow light in various solvents. In nonpolar solvents, the fluorescence spectra are composed of two bands [maxima of the bands (λ_{fl}) are given in Table 1]. Dyes **4** and **5** demonstrated a strong fluorescence response in nonpolar solvents. As predicted by the Strickler–Berg equation,[22] the fluorescence quantum yields (Φ_{fl}) of heterocycles **4** and **5** decrease in polar solvents as a result of the bathochromic shift in the emission band, which induced both an increase in the rate of nonradiative decay and

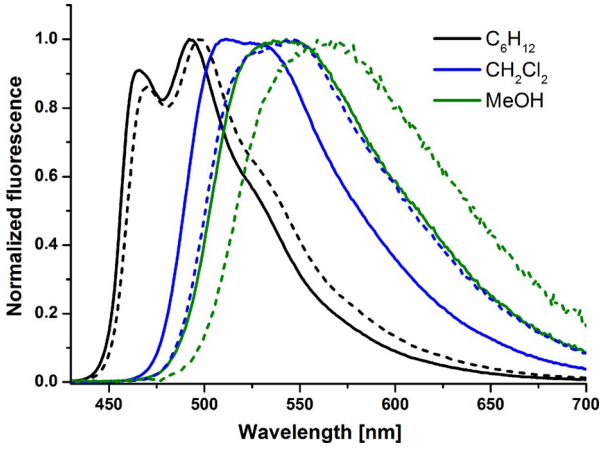


Figure 3. Normalized fluorescence spectra of compounds **4** and **5** in cyclohexane, dichloromethane, and methanol.

Table 1. Photophysical properties of compounds **4** and **5**.

Compd	Solvent	λ_{abs} [nm]	ε [10^{-3} M^{-1} cm^{-1}]	$\Delta\nu$ [cm^{-1}]	λ_{em} [nm]	Φ [%][a]
4	C_6H_{12}	428	4.7	2100	470/498	75
	n-nonane	427	nd	2100	469/497	68
	toluene	436	11.4	3000	502/524	45
	CH_2Cl_2	443	12.7	4300	546	18.5
	MeCN	443	12.8	4500	552	6.5
	MeOH	449	12.6	4400	559	2.5
5	C_6H_{12}	426	15.3	2000	466/492	94
	n-nonane	426	nd	2000	465/493	80
	toluene	436	19.1	2700	494/515	77
	CH_2Cl_2	443	22.6	3000	511	61
	MeCN	443	20.2	3450	523	37
	MeOH	447	18.1	3800	539	11.5

[a] Relative fluorescence quantum yields were determined by using perylene in cyclohexane as the reference. nd: not determined.

a reduction in the rate of radiative decay. Notably, fluorescence quantum yields are higher for isomer **5** than for isomer **4**. The values of the Stokes shifts for compounds **4** and **5** in cyclohexane and *n*-nonane, respectively, were very similar; however, with the increase in solvent polarity, the Stokes shift tended to increase (Figure 4, Table 1). This observation may be attributed to charge redistribution that occurred during the excitation process, which formed the excited state with higher charge separation in chromophores **4** and **5**. Polar solvents decreased the energy of the excited state, which resulted in the increased Stokes shift. For dye **4** in polar solvents, the values were all noticeably higher.

The presence of a cyano group at a peripheral position relative to the aminopyrrole moiety has a non-negligible effect on the optical behavior: the absorption band in hexane is bathochromically shifted versus that of the parent heterocycle prepared from 2,3-dichloroquinoxaline and DBU[21] by approximately 20 nm. However, the emission is not shifted: it is located at approximately $\lambda = 470$ nm in all three cases. The presence of the cyano group has a tremendous effect on Φ_{fl} and

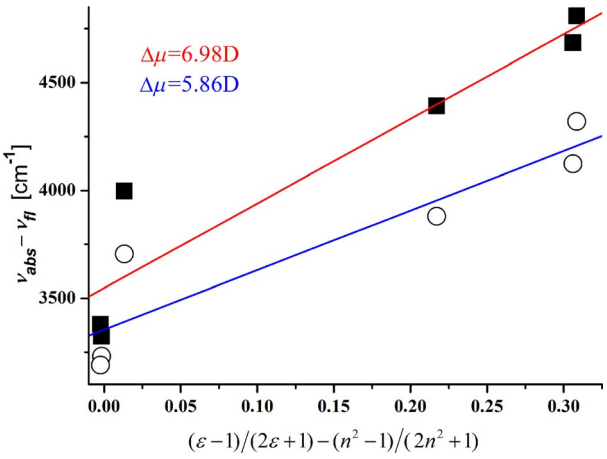


Figure 4. Plot of the spectral shift ($\Delta\nu$) as a function of solvent parameter. **4** (■), **5** (○). Linear fit to the experimental data for isomer **4** (red line) gave $\Delta\mu = 6.98$ D; for isomer **5** (blue line) gave $\Delta\mu = 5.86$ D.

on solvatofluorochromism. Whereas Φ_{fl} for a series of previously studied pyrrolo[2,3-*b*]quinoxalines was approximately 40% in nonpolar solvents,[20] for heterocycles **4** and **5** it was equal to 75 and 94%, respectively. A previously studied DBU-fused pyrrolo[2,3-*b*]quinoxaline[20a] displayed relatively weak solvatofluorochromism ($\Phi_{fl} = 8$% in MeOH), whereas for compounds **4** and **5** their emission intensities decreased more than one order of magnitude.

The Stokes shift values between the absorption and fluorescence spectra can be analyzed with the Lippert–Mataga equation [Eq. (1)]:[23,24]

$$\nu_{abs} - \nu_{fl} = \left(\nu_{abs}^{vac} - \nu_{fl}^{vac}\right) + \frac{2(\mu_e - \mu_g)^2}{4\pi\varepsilon_0 hca^3}\left(\frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}\right) \quad (1)$$

in which $\Delta\nu = \nu_{abs} - \nu_{fl}$ is the solvatochromic Stokes shift, $\Delta\mu = \mu_e - \mu_g$ is the change in the electric dipole moment in the electronic excited (S_1) and ground (S_0) states of the molecule, h is the Planck constant, ε_0 is the vacuum permittivity, c is the velocity of light, a is the radius of the Onsager cavity, ε is the dielectric constant, and n is the refractive index of the solvent. From the plots of the linear dependence of the Stokes shift as a function of the solvent parameter $(\varepsilon-1)/(2\varepsilon+1)-(n^2-1)/(2n^2+1)$, which are presented in Figure 4, we calculated slope values of (3924 ± 708) and (2764 ± 455) cm^{-1} for **4** and **5**, respectively. Assuming the same cavity radius for both isomers, $a = 5$ Å, we obtained $\Delta\mu = 6.98$ and 5.86 D for **4** and **5**, respectively.

Fluorescence decay curves were measured by the "time correlated" single photon counting technique. The curves in nonpolar solvents were well fitted to a single-exponential dependence (the calculated decay times, τ_{fl}, are collected in Table 2), but in polar solvents they appeared to follow at least a two-exponential function. Taking into account these data and the fluorescence quantum yields, we calculated the radiative rate constants for the $S_1 \rightarrow S_0$ transition (according to the formula: $k_r = \phi_{fl}/\tau_{fl}$), which are listed in Table 2. Notably, the experimentally determined rate constant k_r is higher for isomer **5** than for **4**. This observation is consistent with the fact that the absorp-

Table 2. Fluorescence decay times (τ_{fl}) and radiative rate constants (k_r).[a]

Compound	Solvent	τ_{fl} [ns]	k_r [10^7 s^{-1}]
	cyclohexane	8.1	9.2
4	*n*-nonane	7.4	9.2
	toluene	5.8	7.8
	cyclohexane	6.5	14.4
5	*n*-nonane	6.4	12.5
	toluene	6.1	12.7

[a] Calculated according to the formula: $k_r = \phi_{fl}/\tau_{fl}$.

tion extinction value for isomer **5** is higher than that for **4** (**5**: $\varepsilon = 19\,100$ vs. **4**: $\varepsilon \cdot = 11\,400$, in toluene). Slightly stronger solute–solvent dependence for isomer **4** (especially in weakly polar toluene, $\mu = 0.36$ D) may be an indication of its more polar character relative to that of isomer **5** (see theoretical considerations below).

To better understand the photophysical properties of the investigated compounds, we performed quantum-chemical calculations by using the DFT and time-dependent (TD) DFT B3LYP/6-31G(d,p) methods. Some important results of these calculations are displayed in Table 3. The wavelengths (λ) and oscillator strengths (f) for the absorption $S_0 \rightarrow S_1$ (λ_{abs}) and the fluorescence emission $S_0 \rightarrow S_1$ (λ_{fl}) in *n*-nonane solvent were obtained for the structures optimized in the S_0 and S_1 states. The Stokes shifts ($\Delta\nu$) and the dipole moment changes ($\Delta\mu$) were obtained by using the following formulae [Eqs. (2) and (3)]:

$$\Delta\nu = \frac{1}{\lambda_{abs}} - \frac{1}{\lambda_{fl}} \quad (2)$$

$$\Delta\mu = \mu_1 - \mu_0 \quad (3)$$

According to the calculations, dye **5** was characterized by considerably higher oscillator strength (f) than compound **4** (**5**: $f = 0.4266$ vs. **4**: $f = 0.1954$) in absorption as well as in emission. This result correlated well with the experimentally determined larger extinction coefficient (ε) and larger rate constant of fluorescence (k_r) for isomer **5** than for isomer **4** (**5**: $k_r = 12.7$ ns vs. **4**: $k_r = 7.8$ ns, in toluene). We also noticed a higher calculated dipole moment change ($\Delta\mu$) for isomer **4** than for **5** (**4**: $\Delta\mu = 6.5$ D vs. **5**: $\Delta\mu = 5.0$ D), in agreement with experimentally determined values.

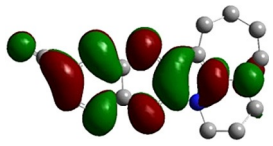
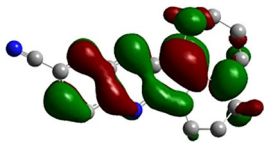
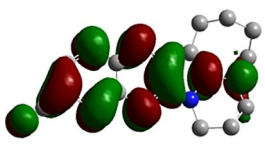
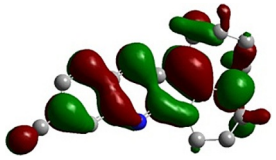
The electronic configurations of the HOMO–LUMO of both isomers, describing the transition between the S_0 and S_1 states, are also presented in Table 3. The shapes of the HOMO and LUMO of both isomers differ depending on the side of the CN group in the compounds. Interestingly, the HOMO of isomer **4** (in the part containing the benzonitrile moiety) resembles the orbital of the benzonitrile molecule of the symmetry a_2 (in the symmetry group C_{2v}), whereas the HOMO of isomer **5** is similar to the orbital b_1 of benzonitrile. Orbitals for the LUMO of both isomers **4** and **5**, in the area of the benzonitrile moiety, are well described by the benzonitrile orbital of symmetry b_1.

On the basis of the orbitals presented in Table 3, we concluded that the differences in the optical properties between isomers **4** and **5** are consequences of the different shapes of the HOMO orbitals in the benzonitrile part of the molecule. In Table S1 and Figure S1 in the Supporting Information, we show that the different shapes of the HOMOs of both isomers find reflection in the extinction ratio between the first ($\lambda \approx 420$ nm) and the second ($\lambda \approx 295$ nm) absorption bands. This ratio is considerably smaller for experimentally studied isomer **4** than for isomer **5** (see Figure 2), and the same is observed for calculated isomers **4** and **5**.

The two-photon absorption cross-sections were measured for compounds **4** and **5** by two-photon excited fluorescence in the $\lambda = 700$–900 nm range. In the case of **4**, the two-photon action cross-section was not registered, whereas **5** presented a value of approximately 2.3 GM.

Compound **5** was chosen for tissue imaging of different mouse organs (e.g., brain, lung, liver, kidney, and spleen) to observe their tissue morphology by TPFM. One advantageous feature of compound **5** is that it can be excited at $\lambda = 900$ nm, which can reduce the autofluorescence in TPFM tissue imaging while also permitting a deeper penetration depth.[25] Figure 5 shows that all of the tested organs were stained clearly and uniformly at a depth of 100 μm or deeper (if a higher laser power could be applied). The close-up image of brain provides the high-resolution morphology of neurons along the hippocampus (Figure 5 f). Furthermore, the brighter spots that

Table 3. Results of the quantum chemistry calculations for isomers **4** and **5**.

Compd	λ_{abs} [nm] S_0	f	μ_0 [D]	λ_{fl} [nm] S_1	f	μ_1 [D]	$\Delta\nu$ [cm^{-1}]	$\Delta\mu$[D]	LUMO	HOMO
4	418.0	0.1954	13.4	538.2	0.0944	19.9	5343	6.5		
5	414.6	0.4266	13.2	505.9	0.2366	18.2	4353	5.0		

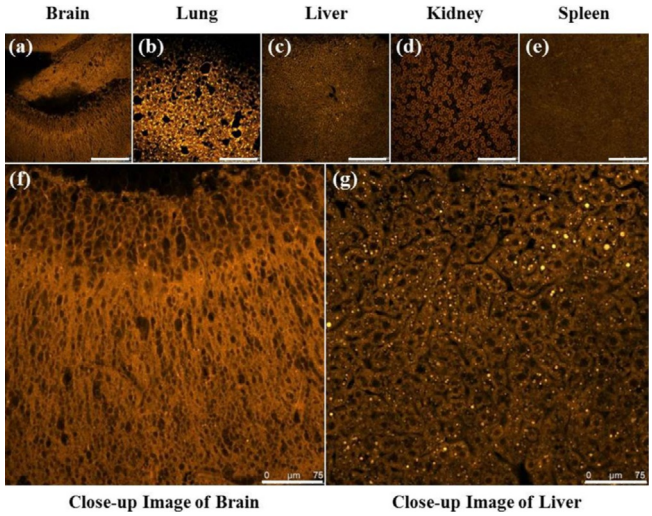


Figure 5. Two-photon microscopy tissue imaging of five different mouse organs stained by compound **5** (10 μM). All the images were captured at a depth of approximately 100 μm of the sectioned tissues (thickness ~400 μm). λ_{ex} = 900 nm; emission filter = 500–605 nm. Images show sectioned tissue images of a) brain, b) lung, c) liver, d) kidney, and e) spleen. Close up images of f) brain and g) liver. Laser power was 28.3 mW at the focal point. Scale bar in panels a–e is 250 μm and for panels f and g is 75 μm.

spread out throughout the high-resolution liver tissue image suggest sublocalization of the compound (Figure 5g).

Conclusions

In conclusion, we showed that replacing a hydrogen atom by an electron-withdrawing cyano substituent led to substantial variations in the optical properties of pyrrolo[2,3-*b*]quinoxalines. This effect was particularly visible in stronger solvatofluorochromism and higher fluorescence quantum yields. The combined effects of 6-cyanoquinoxaline as an electron-deficient moiety and a bridged and fully conjugated tertiary amino group led to absorption of violet photons and emission of green-yellow light, depending on the solvent polarity. As a consequence of the different shapes of the HOMO orbitals in the benzonitrile part of the molecule, depending on the position of the cyano group in the target dye molecules, their spectroscopic properties were different. The new push–pull molecules showed marginal but sufficient two-photon brightness. One of these dyes was successfully applied for two-photon tissue imaging of mouse organs, and it afforded high-resolution tissue morphology of brain hippocampus neurons and sublocalization of the compound in liver tissue.

Experimental Section

General methods

All chemicals were used as received unless otherwise noted. Reagent-grade solvents (i.e., CH_2Cl_2, hexane, toluene) were distilled prior to use. All reported ^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian 500 MHz spectrometer. Chemical shifts (δ) were determined with Me_4Si as the internal reference; *J* values are given in Hz. Chromatography was performed on silica gel (Kieselgel 60, 200–400 mesh).

Procedure for the synthesis of 4 and 5

A sealed tube containing 2,3-dichloroquinoxalin-6-carbonitrile (**2**; 1.6 g, 7 mmol) and 1,8-diazobicyclo[5.4.0]undec-7-ene (3.2 g, 21 mmol) was placed in a preheated oil bath at 115 °C. After 25 min, the mixture was cooled to RT. Then, CH_2Cl_2 was added and column chromatography on silica gel (CH_2Cl_2/acetone = 97:3) was performed to obtain the pure products as orange crystals. Yields: 48% (for **4**), 9% (for **5**).

Characterization data

10-Carbonitrile-2,3,4,5,6,7-hexahydro-1*H*-3a,8,13,13b-tetraazabenzo[*b*]cyclohepta[1,2,3-*jk*]fluorene (**4**): R_f = 0.66 (SiO_2; CH_2Cl_2/EtOAc = 9:1); m.p. 265–266; ^{1}H NMR (500 MHz, $CDCl_3$, Me_4Si): δ = 8.29 (d, *J* = 1.8 Hz, 1H), 7.91 (d, *J* = 8.5 Hz, 1H), 7.53 (dd, *J* = 8.5, 1.8 Hz, 1H), 4.18 (t, *J* = 6.0 Hz, 2H), 3.45 (m, 4H), 2.95 (t, *J* = 6.0 Hz, 2H), 2.28 (m, 2H), 2.00 (m, 2H), 1.87 ppm (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$, Me_4Si): δ = 21.7, 23.0, 26.9, 29.5, 38.1, 49.8, 56.4, 90.3, 108.1, 119.7, 125.0, 128.5, 132.8, 139.4, 140.2, 142.7, 145.2, 153.0 ppm; HRMS (EI): *m/z*: calcd for $C_{18}H_{17}N_5$: 303.1484 [M^+]; found: 303.1484.

11-Carbonitrile-2,3,4,5,6,7-hexahydro-1*H*-3a,8,13,13b-tetraazabenzo[*b*]cyclohepta[1,2,3-*jk*]fluorene (**5**): R_f = 0.20 (SiO_2; CH_2Cl_2/EtOAc = 9:1); m.p. 256–257; ^{1}H NMR (500 MHz, $CDCl_3$, Me_4Si): δ = 8.22 (d, *J* = 1.8 Hz, 1H), 7.96 (d, *J* = 8.5 Hz, 1H), 7.60 (dd, *J* = 8.5, 1.8 Hz, 1H), 4.18 (t, *J* = 6.0 Hz, 2H), 3.47 (m, 4H), 2.97 (t, *J* = 6.0 Hz, 2H), 2.29 (m, 2H), 2.00 (m, 2H), 1.88 ppm (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$, Me_4Si): δ = 21.7, 22.9, 26.8, 29.5, 38.00, 49.8, 56.23, 91.1, 105.7, 120.0, 126.9, 128.1, 133.1, 136.1, 142.7, 143.4, 145.2, 153.9 ppm; HRMS (EI): *m/z*: calcd for $C_{18}H_{17}N_5$: [M^+] 303.1484; found: 303.1484.

Optical properties

The absorption and fluorescence spectra of dyes **4** and **5** in liquid solutions of cyclohexane, *n*-nonane, toluene, dichloromethane, acetonitrile, and methanol (all spectroscopic grade) were measured at room temperature with the aid of a PerkinElmer UV/Vis Lambda 35 absorption spectrometer and a Hitachi F-7000 fluorescence spectrometer, respectively. Fluorescence quantum yields (Φ_{fl}) were determined with respect to perylene in cyclohexane (Φ_{fl} = 0.94).[26] The error inherent with the Φ_{fl} estimation did not exceed 10%.

Fluorescence spectra of both compounds in *n*-nonane matrix at 5 K were monitored with the aid of a home-built spectrometer equipped with a McPherson 207 monochromator, an EMI9659 photomultiplier, and an EasyScan PC module.

Fluorescence kinetics studies were performed with the aid of the "time correlated" single photon counting technique (in the inverted time mode). Excitation pulses were provided by the second harmonics of a mode-locked Coherent Mira-HP femtosecond laser pumped by a Verdi 18 laser. Original repetition rate of a Mira laser was reduced with the aid of an APE Pulse selector to 2 MHz. Fluorescence photons were dispersed with a McPherson 207 monochromator and detected with a HMP-100–50 hybrid detector and SPC-150 module inserted into a PC, both from Becker&Hickl GmbH. Fluorescence decays were long relative to the detected time width of the excitation pulses (≈200 ps), and therefore, the decay curves (delayed by 1 ns with respect to the excitation) were fitted to

single or double exponential dependences without using a deconvolution procedure. Estimated precision of the decay time determination was 10 ps.

The two-photon absorption measurements were performed by using an IsoPlane SCT 320 fluorescence spectrometer (Princeton Instrument) equipped with an RMS4X-PF (Olympus) objective lens by using methods described earlier.[27] Samples were measured in 1.0×1.0 cm quartz cuvettes in a 90° setup with direct excitation from a femtosecond (<100 fs) titan-sapphire-laser (Chameleon Compact OPO-Vis, Coherent) with 80 MHz repetition rate. The measurements were performed in the wavelength range from 740 to 880 nm. Details of the calculations are given in the Supporting Information.

Calculations

All calculations within this work were made with the aid of the Gaussian 09 package.[28] Optimization of the molecular geometry in the electronic ground (S_0) and lowest excited (S_1) states were performed with the DFT and TD DFT B3LYP/6-31G(d,p) methods. The spectra of molecules in solutions were calculated within the framework of the PCM model with the use of the default options of this model implemented in the Gaussian package. The vibrational structures of the electronic spectra were calculated with a procedure included in Gaussian 09, which used the Franck–Condon factors and the Duchinsky matrix.

Tissue imaging for organs of mice

A C57L6-type mouse (6 weeks, male) was used for this experiment. Experiments were done under light-protected conditions, in a dark-room, and with the use of aluminum foil. The mouse was dissected after dislocation of the cervical vertebral. Blood perfusion with phosphate-buffered saline (PBS, 1X solution) was performed for elimination of blood. The organs were dissected, washed with PBS buffer, and then sliced with a vibrating blade microtome (VT1000S, Leica, Germany) with 400 μm thickness. Tissue slice samples were immersed in 4% aqueous paraformaldehyde for 1 h to fix the tissue. After the fixation, the tissues were immersed in the dye solution (10 μM) for 30 min at 37 °C. Stained samples were placed on the glass slide for imaging, after washing with PBS several times. Two-photon microscopy equipped with a Ti-Sapphire laser (Chameleon Vision II, Coherent) at 140 fs pulse width and 80 MHz pulse repetition rate was used (TCS SP5 II, Leica, Germany). The excitation laser was tuned to $\lambda = 900$ nm and emission light was collected at $\lambda = 500$–605 nm in the case of compound **5**. Prepared tissue slice samples were mounted on the tight-fitting holder. The power of the excitation laser was approximately 28.3 mW at the focal point. The imaging resolution was 1024×1024 pixels and scanning speed was 200 Hz all the way through the imaging. Acquired images were processed by using LAS AF Lite (Leica, Germany).

CCDC 1456392 (**4**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre.

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