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Excited State Intramolecular Proton Transfer and Photophysics of a New Fluorenyl Two-Photon Fluorescent Probe

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Abstract

The steady-state photophysical, NMR, and two-photon absorption (2PA) properties of a new fluorene derivative (**1**) containing the 2-(2'-hydroxyphenyl) benzothiazole (HBT) terminal construct was investigated for use as a fluorescence probe in bioimaging. A comprehensive analysis of the linear spectral properties revealed inter- and intramolecular hydrogen bonding and excited state intramolecular proton transfer (ESIPT) processes in the HBT substituent. A specific electronic model with a double minimum potential energy surface was consistent with the observed spectral properties. The 2PA spectra were obtained using a standard two-photon induced fluorescence method with femtosecond kHz laser system, affording a maximum 2PA cross section of ~600 GM, a sufficiently high value for two-photon fluorescence imaging. No dependence of two-photon absorption efficiency on solvent properties and hydrogen bonding in the HBT substituent was observed. The potential use of this fluorenyl probe in bioimaging was demonstrated via one- and two-photon fluorescence imaging of COS-7 cells.

Keywords

Fluorescence probes; imaging agents; hydrogen bonding; two-photon induced fluorescence

Introduction

The development of new two-photon absorbing organic molecules with tailored luminescent properties is of continued interest both scientifically and, importantly, because of the diverse variety of current and emerging applications that rely on particular luminescent materials, e.g., two-photon fluorescence (2PF) bioimaging,[1,2] FLIM and STED microscopy,[3–5] 3D optical data storage and two-photon molecular switching,[6–9] and fluorescence sensing.[10,11] The parameters of molecular fluorescence emission can be influenced by numerous inter- and intramolecular processes that occur upon excitation,[12] including excited state intramolecular proton transfer (ESIPT) phenomenon.[13–16] Molecules that undergo ESIPT typically exhibit fast (on the picosecond time scale) proton transfer in the excited electronic state,[14] and have been utilized in UV filtering,[17] sensing,[18] efficient dye lasing,[19]

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and scintillator counting[20] among other applications. Therefore, the combination of efficient two-photon absorption (2PA) and ESIPT properties may open new avenues, facilitated by 2PA fluorescent materials.

2PA fluorene-based molecules are promising for most of the applications mentioned above[21–26] and, especially, for fluorescence bioimaging[2,22–24] and 3D optical data storage.[25,26] A new fluorene derivative was designed and synthesized that contains both diphenylamino and 2-(2'-hydroxyphenyl)benzothiazole (HBT) end substituents. The HBT substituent in this dipolar molecule was designed to test the possibility of ESIPT. Thus, the linear photophysical and 2PA properties of (**1**) are reported herein, with an eye to identify specific fluorescence properties that may be useful for nonlinear optical applications. Effects of inter- and intramolecular hydrogen bonding and ESIPT processes on the linear and nonlinear (two-photon) spectral properties of **1** were elucidated. Fluorescence bioimaging, both under one-photon and two-photon excitation, was performed for COS-7 cells that were incubated with **1**, indicating the potential of this new probe in bioimaging.

Results and Discussion

Synthesis of Fluorescence Probe 1

Probe **1** (Scheme 1) is comprised of a fluorenyl π -electron bridge with an unsymmetrical D- π -A chemical structure (where D and A are electron donor and acceptor moieties, respectively). Scheme 1 illustrates a straightforward strategy to incorporate *o*-hydroxyphenyl-2-benzothiazole (HBT) as an acceptor group in the fluorenyl 2-position. The synthesis of **1** began with the preparation of 2-(benzothiazol-2-yl)-5-vinylphenoxy(*t*-butyl)dimethylsilane, precursor **C**. The condensation of 2-aminobenzenethiol and 4-bromo-2-hydroxybenzoic acid was carried out in the presence of PPSE in *o*-dichlorobenzene, providing **A** in 81% yield.[27] The hydroxyl group of this precursor was then protected with TBDMS ether (*t*-butyldimethylsilyl ether) using *tert*-butylchlorodimethylsilane and imidazole in DMF at 45 °C, affording precursor **B** in 90% yield. In the ¹H NMR spectrum, the methyl protons on α and β carbons relative to Si appeared at approximately 1.02 and 0.4 ppm, respectively, confirming the presence of the TBDMS group.

Pd-catalyzed Stille coupling was subsequently performed between 2-(benzothiazol-2-yl)-5-bromophenoxy(*t*-butyl)dimethylsilane (**B**) and tributyl(vinyl)tin in refluxing THF in a high pressure Schlenk tube with Pd(dppf)Cl₂·CH₂Cl₂ and CuI. Precursor **C** was obtained in 62% isolated yield. The basic media of the Stille coupling reaction promoted partial deprotection of the TBDMS, affording, 2-(benzothiazol-2-yl)-5-vinylphenol (**D**) in 14% yield. Both precursors were characterized by elemental analysis and NMR spectroscopy. ¹H NMR spectra of compounds **C** and **D** displayed the signals of the three mutually coupled nonequivalent vinyl protons (three-spin ABC system). The H_C and H_B protons each produced a doublet at approximately 5.7 and 5.3 ppm, respectively. While the H_A proton appeared as a quartet at ca. 6.7 ppm. The signal characteristic for the OH proton in compound **D** was observed as a singlet at 12.42 ppm.

With the aim of removing the silyl ether protecting group after completion of the Pd coupling reaction, the Pd-catalyzed Heck coupling reaction between 9,9-bis(2-(2-methoxyethoxy)ethyl)-7-iodo-N,N-diphenyl-fluoren-2-amine (**E**) and **C** was performed. The reaction was conducted in a high pressure Schlenk tube with Pd(OAc)₂, tri-*o*-tolylphosphine, and Et₃N in DMF at 90 °C for 40 h (Scheme 1). Interestingly, the basic media of the Heck coupling reaction deprotects the TBDMS group, producing the desired product that contains the free phenol group (fluorescence probe **1**) in 68% yield. In the ¹H NMR spectrum, the resonances at ca. 1.02 and 0.4 ppm disappeared. Instead a resonance at 12.5 ppm was observed, originating from the OH of the phenoxy group, in agreement with

the proposed structure. Elemental analysis and NMR spectroscopy were found to be consistent with the proposed structure of **1**. The easy introduction and removal of the silyl ether protecting group (TBDMS) offers advantages in our synthetic strategy, achieving the final target without an extra deprotection step. The aforementioned synthesis resulted in an efficient strategy to create a potentially useful probe for fluorescence bioimaging.

Linear Spectral Properties

The primary steady-state linear spectral properties of **1** (Figure 1 and Table 1) are similar to that of typical unsymmetrical fluorene derivatives.[28–30] The absorption spectra were nearly independent of solvent polarity, Δf . [12] Practically the same shape and position of the absorption spectrum of **1** observed in aprotic solvents and protic methanol (Figure 1a–d, curves 1), where the intermolecular hydrogen bonding between OH group of HBT substituent in **1** and solvent molecules can occur.[31] The hydrogen bonding was confirmed by NMR (Figure 2). The ^1H NMR spectra were measured in CDCl_3 , CD_2Cl_2 , $\text{DMSO}-d_6$, and methanol- d_3 . In an aprotic solvent like CDCl_3 and CD_2Cl_2 , the HBT moiety in **1** partakes in intramolecular hydrogen bonding, confirmed by a sharp signal at δ 12.0 ppm, assigned to the hydroxyl hydrogen. No significant change in the chemical shifts of the aromatic protons was observed with the addition of CH_3OH to the CDCl_3 solution of **1**. On the other hand, the signal at δ 12.0 ppm, assigned to the hydroxy group decreased drastically with the addition of methanol- d_4 to the CDCl_3 solution, leading us to believe the hydroxyl hydrogen participates in hydrogen bonding with protic solvents such as methanol.

The observed similarity of the steady-state absorption spectra in different solvents suggest only a weak influence of inter- and intramolecular hydrogen bonding on the linear absorption spectra of **1**. These data and analysis, presented earlier,[32,33] presume the absence of proton transfer (PT) processes in the HBT end substituent in the ground electronic state, S_0 , of **1** in the solvents studied here. In the case of PT processes in the S_0 state, the absorption spectra should be sensitive to solvent polarity, which was not observed here.

The steady-state fluorescence spectra of **1** (Figure 1a–d, curves 1', 2') exhibited strong solvatochromic behavior with large Stokes shifts in polar solvents (Table 1). In aprotic media, the values of the Stokes shifts of **1** exceed values for typical unsymmetrical fluorenes that lack the HBT substituent but have comparable changes in the stationary dipole moment upon electronic excitation in the same solvent and similar π -conjugation lengths.[28, 29] This behavior can be explained by excited state intramolecular proton transfer (ESIPT) in the HBT substituent of **1** in aprotic solvents, similar to ones in the discrete HBT molecule under electronic excitation.[32] This suggests that the HBT substituent has the proton predominantly on the phenol oxygen (enol form) in the ground state of **1**, whereas, after excitation, this acidic proton migrates to the benzothiazolyl nitrogen atom (keto form). Intramolecular hydrogen bonding of **1** in aprotic solvents can facilitate these processes in the excited state.

Intermolecular hydrogen bonding occurs between **1** and methanol, as evidenced by analysis of the ^1H NMR spectra (Figure 2) and the corresponding data in reference[31] (i.e., the OH of the HBT moiety and methanol solvent molecules). Presumably, in this case, no ESIPT occurs, which is reflected in the decreased Stokes shift of **1** in methanol relative to the less polar and aprotic ACN (Figure 1d and Table 1).

The fluorescence spectra of **1** in aprotic solvents were independent of excitation wavelength, λ_{exc} . This behavior is typical for the most of the fluorene derivatives reporting that lack intramolecular hydrogen bonding.[28–30] The values of fluorescence quantum yields, Φ , were sufficiently high (0.6–1.0) and exhibited nearly constant spectral dependencies (Figure

3a). Fluorescence lifetimes of **1**, in the aprotic solvents studied here, exhibited a monoexponential decay and increased with solvent polarity (Table 1). The excitation anisotropy spectra, $r(\lambda)$, reflect the nature of the absorption bands of **1** in both protic and aprotic solvents (Figure 3c, d). The electronic structure of **1** in aprotic solvents is characterized by a single electronic transition in the main absorption band at $\lambda_{abs}^{max} \approx 410$ nm. This is deduced from the constant value of anisotropy (Figure 3c) in the spectral range 370–460 nm.[12,28] In viscous pTHF, the fundamental anisotropy value of **1**, $r_0 \approx 0.33$, indicates the angle is ca. 20° between the absorption, $S_0 \rightarrow S_1$, and emission, $S_1 \rightarrow S_0$, transition dipole moments. The excitation anisotropy of diphenylamine-based fluorenes without the HBT group exhibits a nearly parallel orientation of these transition dipoles ($r_0 \approx 0.4$).[34] This suggests that the ESIPT processes that occur in the HBT substituent may lead to a nonparallel orientation of the absorption and emission transitions of **1** in aprotic solvents. In low viscosity media (DMSO, toluene, hexane, ACN) the excitation anisotropy values decrease in accordance with an increase in τ/θ (θ is the rotational correlation time).[12]

In contrast to aprotic solvents, **1**, in methanol, was characterized by a dependence of the fluorescence spectra on λ_{exc} , while no evidence of ESIPT or noticeable spectral dependence of Φ was observed (Figure 3b). In this case, the decay of fluorescence emission exhibited a biexponential process, with the corresponding lifetimes (Table 1). The excitation anisotropy spectra in methanol and viscous glycerol (Figure 3d) revealed a rather complex nature of the main absorption band of **1** in protic media. The values of r are not constant in the spectral range of the main absorption band and strictly depend on λ_{obs} , i.e., additional electronic transitions $S_0 \rightarrow S_n$ ($n = 2, 3, \dots$), located close to $S_0 \rightarrow S_1$, or a complicated potential surface of S_1 state may be present.[35] A comprehensive investigation of the spectral parameters of **1** in methanol and glycerol revealed two main fluorescence and excitation bands (Figure 4a, b). From these spectra, one can see two fluorescence bands with maxima about 405 and 590 nm (Figure 4a), corresponding to the absorption contours revealed by the excitation spectra with maxima at ca. 355 and 410 nm (Figure 4b), respectively.

Based on our data, two different molecular models can be proposed to account for the observed spectral properties. The first model involves the existence of two types of solvated molecules with corresponding absorption ($\lambda_{abs}^{max} \approx 355$ and 410 nm) and emission ($\lambda_{fl}^{max} \approx 405$ and 590 nm) maxima in methanol solution. One form is dominant ($\approx 95\%$ of the total concentration) with $\Phi \approx 0.15$ and $\tau \approx 0.3$ ns while the other minor form ($\approx 5\%$) is highly fluorescent ($\Phi \approx 1.0$) with $\tau \approx 1.5$ ns. The second model one may invoke is based on a double minimum potential surface of the first excited state, S_1 , of **1** in protic solvent, i.e., methanol (Figure 4c), which leads to the double band fluorescence emission and spectral dependence of Φ . The following one-photon nonlinear optical measurements were performed to distinguish between these two models.

Saturation of the Fluorescence Emission Under Strong One-Photon Excitation

In order to check the validity of the first model, the effects of fluorescence saturation of **1** in methanol at the short and long wavelength emission bands were investigated under powerful excitation at 355 nm (Figure 4d). In the case of two different types of solvated molecules in the S_0 state of **1** (model two), fluorescence saturation processes should be different at 405 nm and 590 nm due to strong differences in the electronic parameters of these two species and their concentrations. As follows from the obtained dependences, the fluorescence saturation of the short and long wavelength emission are very close (Figure 4d), indicative of a single type of absorbing molecule in the ground state. Thus, the second proposed model, with the double minima potential surface of S_1 state (Figure 4a), is more probable.

2PA Spectral Properties

The unsymmetrical fluorene **1** exhibited two well defined 2PA bands (Figure 5). One band is less efficient, with a 2PA cross section $\delta_{2PA} \sim 150\text{--}200$ GM and overlapped with the long wavelength linear absorption peak. That means two-photon transitions are not strictly forbidden by selection rules in the primary one-photon allowed absorption band. The second 2PA peak at ~ 640 nm is two-photon allowed and exhibited a 2PA cross section $\sim 500\text{--}550$ GM. It should be mentioned that the electronic transition revealed by the minimum in the excitation anisotropy spectra at ~ 300 nm (Figure 3c, d) belongs to the absorption of diphenylamino substituent of **1**[36] and is not associated with the short wavelength 2PA band at 640 nm.[37] The spectral shape and absolute values of δ_{2PA} were nearly independent of polarity and pH properties of solvent (Figure 5, curves 1–3). This is indicative of a minor role of inter- and/or intramolecular hydrogen bonding of **1** in the 2PA process. A complex structure of the S_1 state and/or a higher density of electronic transitions of **1** in methanol did not result in increased 2PA cross sections.

One- and Two-Photon Fluorescence Bioimaging

The use of fluorescence probe **1** as a 2PA biological contrast agent was evaluated using a COS-7 cell line. Obtained images (Figure 6) show the localization of the dye in the cytoplasm of the cells, indicating potentially preferential staining with this fluorophore for cytoplasmic components. Furthermore, probe **1** did not undergo noticeable photobleaching during continuous exposure to UV excitation light. This is consistent with results obtained from comprehensive photochemical studies of other similar fluorene-based compounds under both one- and two-photon excitation in solvents of varying polarity.[38–41] Two-photon fluorescence was observed predominantly from the cytoplasmic region (Figures 6 panel c, d), consistent with the images collected from one-photon fluorescence imaging. The dye appears to be selective for an organelle surrounding the nuclei, possibly the mitochondria. Typically in COS-7 cells the mitochondria are generally localized around one hemisphere of the cell nucleus, as is observed in the micrographs. Currently, colocalization studies are being carried out to ascertain the dye's mitochondria specificity. As a stereograph is more visual than a planar view, COS-7 cells which incubated with fluorophore **1** were scanned slice by slice in confocal mode, affording 3D image (Figure 6c). This technique should prove useful when detailed studies of the cell phenotype are being carried out, as it will allow one to visualize small cell features relying on the intrinsic resolution of upconverted fluorescence.

Conclusions

A new fluorene-based fluorescent probe (**1**) was synthesized that is capable of either intramolecular or intermolecular hydrogen bonding, depending on the environment (aprotic or protic). A comprehensive analysis of linear photophysical and nonlinear optical properties of this new probe was accomplished, and specific spectral properties were presented. The steady-state absorption, fluorescence, and NMR spectra of **1** revealed intramolecular hydrogen bonding and ESIPT processes in aprotic solvents of differing polarity. Strong solvatochromic effects of the fluorescence emission can be explained by ESIPT. In protic methanol efficient intermolecular hydrogen bonding with solvent molecules interrupted proton transfer processes in the HBT substituent of **1** and a spectral dependence of fluorescence emission and the corresponding quantum yield as a function of excitation wavelength were observed. The results of one-photon fluorescence saturation measurements suggest a homogeneously solvated molecular ensemble in protic solvent (methanol). A specific double minimum potential energy surfaces of the S_1 electronic excited state was proposed and consistent the observed spectral properties.

2PA spectra of **1** were obtained over a broad spectral range, and exhibited two well defined absorption bands at 640 and 840 nm with the maximum 2PA cross sections at ca. 500–550 GM and 150–200 GM, respectively. The value of δ_{2PA} was not solvent dependent. A long wavelength 2PA band at 840 nm was sufficiently intense, overlapping nicely with the tuning range of commercially available femtosecond lasers, an important aspect for practical applications of **1**, particularly in multiphoton fluorescence microscopy. To demonstrate the potential utility of **1** as a probe, one- and two-photon fluorescence imaging of COS-7 cells incubated with probe **1** was conducted. Thus, probe **1**, with its high fluorescence quantum yield, high photostability, efficient 2PA, hydrogen bonding, and the sensitivity of ESIPT to the surrounding media, is a promising new contrast agent for advanced nonlinear optical applications, including multiphoton fluorescence bioimaging.

Experimental Section

Materials and Synthetic Procedures

Pd-catalyzed reactions were performed in high pressure Schlenk tubes under N₂ atmosphere. 4-Bromo-2-hydroxybenzoic acid was purchased from Trans World Chemicals, Inc. and used without further purification. THF was distilled under Ar over sodium benzophenone ketyl. 2-Aminothiophenol was distilled under reduced pressure before use. All other reagents and solvents were used as received from commercial suppliers. The ¹H and ¹³C NMR spectroscopic measurements were performed using a Varian 500 NMR spectrometer at 500 MHz with tetramethylsilane (TMS) as internal reference; ¹H (referenced to TMS at $\delta = 0.0$ ppm) and ¹³C (referenced to CDCl₃ at $\delta = 77.0$ ppm). Chemical shifts of ¹H and ¹³C spectra were interpreted with the support of CS ChemDraw Ultra version 5.0. Elemental analyses were performed at Atlantic Microlabs.

Synthesis of 2-(benzothiazol-2-yl)-5-bromophenol (A)

4-Bromo-2-hydroxybenzoic acid (5 g, 23.05 mmol), 2-aminothiophenol (3.45 g, 27.63 mmol), and *o*-dichlorobenzene (90 mL) were placed in a 250 mL 3-necked flask. Polyphosphoric acid trimethylsilyl ester (PPSE, 28 g) was added. After addition of PPSE the reaction mixture turned from white to bright yellow. The reaction mixture was stirred at room temperature for 20 min and then heated at 90 °C under N₂ for 36 h. TLC using hexane/EtOAc (3:2) was utilized to monitor the reaction progress. After 20 h the reaction mixture was completed and allowed to cool to room temperature. The reaction mixture was poured into water and extracted with CH₂Cl₂. The organic phase was washed with a 5% NaOH solution than a saturated solution of NaCl. The extract was dried over anhydrous MgSO₄ and concentrated under reduced pressure. *o*-Dichlorobenzene was removed by vacuum distillation, affording a pale yellow solid. Purification was carried out by silica gel column chromatography using CH₂Cl₂ as eluent to afford a white solid 5.7 g (81 % yield), m.p. 142–143 °C (lit. 134–135 °C).^[27] ¹H NMR (500 MHz, CDCl₃) δ : 12.71 (s, 1H, Ph-OH), 7.99 (d, 1H, Ph-H); 7.91 (d, 1H, Ph-H); 7.53 (t, 1H, Ph-H), 7.44 (t, 1H, Ph-H); 7.29 (d, 2H, Ph-H); 7.09 (d, 1H, Ph-H) ppm. ¹³C NMR (500 MHz, CDCl₃) δ : 168.7 (Ar-C=N), 158.7 (Ph-OH), 151.8, 132.3, 129.2, 126.8, 126.4, 125.8, 122.8, 122.2, 121.5, 121.1, 116.1 ppm.

Protection of 2-(benzothiazol-2-yl)-5-bromophenol (B)

A DMF (5 mL) solution of 2-(benzothiazol-2-yl)-5-bromophenol (1 g, 3.26 mmol), tert-butyltrimethylsilyl chloride (1.08 g, 7.17 mmol), and imidazole (0.97 g, 14.24 mmol) was stirred at 40 °C under N₂ atmosphere for 18 h. After the reaction mixture was diluted with CH₂Cl₂ (150 mL) and washed with water, the organic phase was dried over Na₂SO₄, filtered, and the solvent was removed under reduced pressure to yield a yellow oil. The crude product was purified by silica gel column chromatography using hexane/THF (4:1) as eluent, affording 1.24 g of a white solid (90% yield), m.p. 84–85 °C, ¹H NMR (500 MHz,

CDCl₃) δ : 8.28 (d, 1H, Ph-H); 8.08 (d, 1H, Ph-H); 7.94 (d, 1H, Ph-H); 7.49 (t, 1H, Ph-H); 7.38 (t, 1H, Ph-H); 7.24 (d, 2H, Ph-H); 7.16 (s, 1H, Ph-H); 1.02 (bs, 9H, CH₃); 0.40 (bs, 6H, CH₃) ppm. ¹³C NMR (500 MHz, CDCl₃) δ : 165.9 (Ar-C=N), 157.6 (Ph-O-Si), 155.4, 139.1, 134.3, 129.3, 128.1, 128, 127.9, 126.7, 126.1, 126, 125.9, 124.6, 29.4 (CH₃), 22.2 (Si-CH₃), 3.2 (Si-CH₃) ppm. Anal. Calcd. for C₁₉H₂₂BrNOSSi: C, 54.28; H, 5.27; N, 3.33; S, 7.63. Found: C, 54.34; H, 5.28; N, 3.25; S, 7.65.

Synthesis of 2-(benzothiazol-2-yl)-5-vinylphenoxy(*t*-butyl)dimethylsilane (C)

2-(Benzothiazol-2-yl)-5-bromophenoxy(*t*-butyl)dimethylsilane (0.6g, 1.42mmol), Pd(dppf)Cl₂.CH₂Cl₂ (0.1 g, 0.14 mmol) and CuI (0.05 g, 0.26 mmol) were transferred to a high pressure Schlenk tube, and the liquid reagents THF (14 mL) and tributyl(vinyl)tin (0.58 g, 1.84 mmol) added. The Schlenk tube was then sealed and stirred at the appropriate temperature. After 48 h silica gel TLC indicated disappearance of aryl bromide, the dark brown reaction mixture was cooled to room temperature, diluted CH₂Cl₂, and filtered through a plug of silica gel. The crude product was concentrated and purified directly by flash chromatography on silica gel using hexane/CH₂Cl₂, (4:1) providing 0.32 g of white solid (62 % yield). m.p. 62–63 °C, ¹H NMR (500 MHz, CDCl₃) δ : 8.38 (d, 1H, Ph-H); 8.08 (d, 1H, Ph-H); 7.93 (d, 1H, Ph-H); 7.48 (t, 1H, Ph-H); 7.35 (t, 1H, Ph-H); 7.15 (d, 1H, Ph-H); 7.03 (s, 1H, Ph-H); 6.70 (q, 1H, Ph-CH=CH₂); 5.78 (d, 1H, Ph-CH=CH₂); 5.35 (d, 1H, Ph-CH=CH₂); 1.03 (s, 9H, CH₃); 0.41 (s, 6H, CH₃) ppm. ¹³C NMR (500 MHz, CDCl₃) δ : 166.54 (Ar-C=N), 157.24 (Ph-O-Si), 155.43, 143.78 (*sp*² carbon of Ph-CH=CH₂), 139.24, 139.03, 133.23, 129.05, 127.68, 126.82, 125.88, 124.41, 122.54, 120.21, 118.66 (*sp*² carbon of Ph-CH=CH₂), 29.37 (CH₃), 22.05 (Si-CH₃), 3.13 (Si-CH₃) ppm. Anal. Calcd. for C₂₁H₂₅NOSSi: C, 68.62; H, 6.86; N, 3.81; S, 8.72. Found: C, 68.65; H, 6.88; N, 3.83; S, 8.70.

Synthesis of 2-(benzo[d]thiazol-2-yl)-5-vinylphenol (D)

A white solid isolated as the minor product from the Stille reaction of **B** (14 % yield). m.p. 132–133 °C, ¹H NMR (500 MHz, CDCl₃) δ : 12.52 (s, 1H, Ph-OH); 7.99 (dd, 2H, Ph-H); 7.65 (d, 1H, Ph-H); 7.52 (t, 1H, Ph-H); 7.42 (t, 1H, Ph-H); 7.13 (s, 1H, Ph-H); 7.03 (d, 1H, Ph-H); 6.71 (q, 1H, Ph-CH=CH₂); 5.88 (d, 1H, Ph-CH=CH₂); 5.38 (d, 1H, Ph-CH=CH₂) ppm. ¹³C NMR (500 MHz, CDCl₃) δ : 168.9 (Ar-C=N), 158.1 (Ph-OH), 151.8, 142.0 (*sp*² carbon of Ph-CH=CH₂), 136.0, 132.5, 128.6, 128.5, 126.6, 125.4, 122.1, 121.5, 117.7 (*sp*² carbon of Ph-CH=CH₂), 116.2, 115.3 ppm. Anal. Calcd. for C₁₅H₁₁NOS: C, 71.12; H, 4.38; N, 5.53; S, 12.66. Found: C, 71.10; H, 4.31; N, 5.36; S, 12.64.

Synthesis of 9,9-bis(2-(2-methoxyethoxy)ethyl)-2,7-diiodo-fluorene (F)

2,7-Diiodofluorene (2 g, 4.78 mmol), 1-bromo-2-(2-methoxy-ethoxy)ethane (2.09 g, 11.46 mmol), and KI (0.08 g, 0.48mmol) were placed into DMSO at rt. To the stirred solution, freshly powdered KOH (1.0 g, 17.9 mmol) was slowly added, turning the yellow reaction mixture dark green. The reaction was followed to completion via TLC hexanes/EtOAc (3:2). The reaction mixture was poured into distilled water and its organic components extracted with CH₂Cl₂, and dried over MgSO₄. The resulting mixture was filtered and concentrated to give dark orange oil which was purified via silica gel column chromatography using the same eluent system used to monitor the reaction on TLC. Compound **F** was obtained as a light yellow oil (1.6. g, 55% yield). ¹H NMR (500 MHz, CDCl₃) δ : 7.74, (d, 2H, Ph-H); 7.6 (dd, 2H, Ph-H); 7.4 (d, 2H, Ph-H); 3.39 (t, 10H, OCH₂, OCH₃); 3.19 (q, 4H, OCH₂); 2.77 (t, 4H, CH₂); 2.36 (t, 4H, OCH₂) ppm. ¹³C NMR (500 MHz, CDCl₃) δ : 150.7, 139.1, 136.6, 136.4, 132.5, 132.4, 121.6, 121.4, 93.3 (C-I), 93.2 (C-I), 71.7, 69.9, 66.7, 59.1 (OCH₃), 51.6 (C9), 39.4 (CH₂) ppm. Anal. Calcd. for C₂₃H₂₈I₂O₄: C, 44.39; H, 4.54. Found: C, 44.24; H, 4.61.

Synthesis of 9,9-bis(2-(2-methoxyethoxy)ethyl)-7-iodo-N,N-diphenyl-fluoren-2-amine (G)

Iodofluorene intermediate **E** (1 g, 1.6 mmol) was dissolved in 7 mL of 1,2-dichlorobenzene at room temperature under N₂. To this was added K₂CO₃ (1.77 g, 12.8 mmol), 18-crown-6 (0.12 g, 0.45 mmol), and copper bronze (0.50 g, 7.87 mmol) at room temperature. Diphenylamine (0.31 g, 1.8 mmol) was added, and the mixture was degassed. The reaction mixture was heated to 180 °C under nitrogen for 28 h. Upon completion, the brown mixture was filtered through a short silica gel plug and the yellow solution was concentrated resulting in brown-yellow oil. 1,2-Dichlorobenzene was removed under reduced pressure. The crude product was further purified by column chromatography on silica gel with CH₂Cl₂/ethyl acetate (4.5:0.5) as the eluent to afford a colorless oil 0.47 g (45% yield). m.p. 99–100 °C, ¹H NMR (500 MHz, CDCl₃) δ: 7.69 (d, 1H, Ph-H); 7.63 (dd, 1H, Ph-H); 7.5 (d, 1H, Ph-H); 7.33 (d, 1H, Ph-H); 7.27 (m, 4H, Ph-H); 7.11 (bd, 5H, Ph-H); 7.04 (m, 3H, Ph-H); 3.35 (bm, 14H, OCH₂, OCH₃); 2.87 (bm, 4H, CH₂); 2.26 (m, 4H, OCH₂) ppm. ¹³C NMR (500 MHz, CDCl₃) δ: 151.3, 149.8, 148.2, 147.7, 139.9, 136.4, 134.0, 132.4, 129.5, 124.5, 123.4, 122.9, 121.0, 120.7, 118.6, 91.5 (C-I), 71.8, 70.0, 67.1, 59.1 (OCH₃), 51.3 (C9), 39.4 (CH₂). Anal. Calcd. For C₃₅H₃₈INO₄: C, 63.35; H, 5.77; N, 2.11. Found: C, 63.48; H, 5.75; N, 2.09.

Synthesis of 5-(2-(9,9-bis(2-(2-methoxyethoxy)ethyl)-2-(diphenylamino)-fluoren-7-yl)vinyl)-2-(benzothiazol-2-yl)phenol (1)

9,9-Bis(2-(2-methoxyethoxy)ethyl)-7-iodo-N,N-diphenyl-fluoren-2-amine (0.28 g, 0.42 mmol), 2-(benzothiazol-2-yl)-5-vinylphenoxy(tert-butyl)dimethylsilane (0.24 g, 0.54 mmol), Pd(OAc)₂ (0.01 g, 0.045 mmol) and P(o-tolyl)₃ (0.027 g, 0.088 mmol) were weighted and transferred to a high pressure Schlenk tube. To this 5 mL of a mixture DMF/Et₃N (5:1) was added. The Schlenk tube was then sealed and stirred at 90 °C for 40 h. The mixture was cooled to room temperature and filtered. The solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel first using ethyl acetate/hexane (4:1), then ethyl acetate/hexane (3.5:2.5) as eluent to afford 0.28 g of a yellow solid (68 % yield). m.p. 140–141 °C, ¹H NMR (500 MHz, CDCl₃) δ: 12.56 (s, 1H, Ph-OH); 7.99 (d, 1H, Ph-H); 7.91 (d, 1H, Ph-H); 7.69 (d, 1H, Ph-H); 7.57 (m, 5H); 7.41 (t, 1H); 7.29 (bm, 16H); 3.35 (m, 14H, OCH₂, OCH₃); 2.88 (m, 4H, CH₂); 2.37 (m, 4H, OCH₂) ppm. ¹³C NMR (500 MHz, CDCl₃) δ: 169.0 (Ar-C=N), 158.1 (Ph-OH), 151.9, 150.6, 149.4, 147.7, 142.1, 140.5, 135.2, 134.6, 132.5, 129.3, 128.8, 128.7, 126.9, 124.3, 123.0, 122.0, 121.5, 120.9, 120.6, 118.7, 118.1, 115.9, 114.9, 71.7, 69.9, 67.2, 59.0 (OCH₃), 51.0 (C9), 39.5 (CH₂) ppm. Anal. Calcd for C₅₀H₄₈N₂O₅S: C, 76.11; H, 6.13; N, 3.55; S, 4.06. Found: C, 76.13; H, 6.15; N, 3.59; S, 4.01. HRMS-ESI theoretical m/z [M+Na]⁺ = 811.317, found 811.315.

One-Photon Spectral Measurements

Linear photophysical properties of new fluorene **1** were investigated in spectroscopic grade aprotic solvents: hexane, cyclohexane, toluene, tetrahydrofuran (THF), dichloromethane (DCM), dimethylsulfoxide (DMSO), acetonitrile (ACN) and protic methanol at room temperature. The steady-state absorption spectra were obtained with Agilent 8453 UV-visible spectrophotometer 10 mm path length quartz cuvettes with dye concentrations $1.5 \cdot 10^{-5} \text{ M} \leq C \leq 3 \cdot 10^{-5} \text{ M}$. The steady-state fluorescence, excitation and excitation anisotropy spectra were measured with PTI QuantaMaster spectrofluorimeter in 10 mm spectrofluorometric quartz cuvettes, with dye concentrations $\sim (1\text{--}1.5) \cdot 10^{-6} \text{ M}$. All fluorescence spectra were corrected for the spectral responsivity of PTI detection system. The fundamental anisotropy values of **1**, r_0 , were obtained in relatively viscous at room temperature solvents, polyTHF (pTHF) and glycerol, where the rotational correlation time, $\theta \gg \tau$ (τ is the fluorescence lifetime of **1**) and observed anisotropy $r = r_0/(1 + \theta/\tau) \approx r_0$. [28] Fluorescence lifetime measurements were performed with a single photon counting system

PicoHarp 300 under linear polarized femtosecond excitation (MIRA 900, Coherent), oriented by the magic angle, with time resolution ≈ 80 ps. The values of fluorescence quantum yields of **1**, Φ , were determined relative to 9,10 diphenylanthracene in cyclohexane as a standard.[12]

Conventional single-photon fluorescence images were obtained using inverted microscope (Olympus IX70) equipped with a QImaging cooled CCD (Model Retiga EXi) and excitation mercury lamp 100 W. In order to improve the fluorescence background-to-image ratios a customized filter cube (Ex 377/50, DM 409, Em 460/50) was used for the one photon fluorescence images. The specifications of the filter cube were tailored to match the excitation wavelength of (**1**) and to capture most of its emission profile.

Besides linear photophysical properties, some spectroscopic peculiarities of **1** were investigated by the measurements of fluorescence intensity saturation under powerful one-photon excitation. The third harmonic of picosecond Nd:YAG laser PL 2143 B Ekspla with excitation wavelength 355 nm, pulse duration ≈ 35 ps (FWHM), pulse energies $\approx 5\text{--}80\mu\text{J}$ and repetition rate 10 Hz was used. Laser beam was focused into the fluorometric quartz cuvette to the waist of radius ≈ 0.5 mm and one-photon induced fluorescence of **1** were detected in the perpendicular direction to the excitation beam by a fiber optic spectrometer USB2000 (Ocean Optics Inc.). No photochemical decomposition of **1** was observed under utilized experimental conditions.

COS-7 Cells Incubation with Fluorescent Probe 1

COS7 cells were plated onto 4-well glass chamber slides. Stock solutions of fluorescent probe **1** dissolved into DMSO were prepared as either 10 or 50 μM solutions. Diluted solutions in complete growth medium, Dulbecco's modification of Eagle's medium (DMEM), were then freshly prepared and placed over the cells for a 1 h period. After incubation, cells were washed with PBS (3–5x) and fixed using 3.7% formaldehyde solution for 5 min at room temperature.

Two-Photon Absorption and Imaging

2PA spectra of **1** were determined in a broad spectral region by typical two-photon induced fluorescence (2PF) method relative to Rhodamine B in methanol and Fluorescein in water (pH=11) as a standards.[42] A PTI QuantaMaster spectrofluorimeter and femtosecond Clark-MXR CPA-2010 laser pumped an optical parametric generator/amplifiers (TOPAS, Light Conversion), with pulse duration, ≈ 140 fs, (FWHM), tuning range 580–940 nm, pulse energies $\leq 0.15\mu\text{J}$ and 1 kHz repetition rate were used. Two-photon fluorescence measurements were performed in 10 mm fluorometric quartz cuvettes with dye concentrations $\sim 3\cdot 10^{-5}$ M in hexane, methanol and ACN. The experimental fluorescence excitation and detection conditions corresponded to the negligible reabsorption processes which can effects 2PA measurements. The quadratic dependence of two-photon induced fluorescence intensity on the excitation power was checked for each excitation wavelength.

Two-photon fluorescence microscopy (2PFM) images were collected on a modified Olympus Fluoview FV300 microscope system coupled to a tunable Coherent Mira 900F Ti:sapphire, 76 MHz, modelocked, femtosecond laser tuned to 730 nm. An emission long-pass filter (cutoff 690 nm) was placed in the microscope scanhead to avoid background fluorescence from the excitation source. Consecutive layers, separated by approximately 0.3 μm , were recorded to create a 3D reconstruction from overlaid two-photon fluorescence images. The two-photon induced fluorescence was collected with a 60x microscope objective (UPLANSAPO 60x, NA= 1.35, Olympus).

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Figure 1.

Normalized absorption (1, 2) and fluorescence (1', 2') spectra of **1** in: a) hexane (1, 1'), cyclohexane (2, 2'); b) toluene (1, 1'), THF (2, 2'); c) CH₂Cl₂ (1, 1'), DMSO (2, 2') and d) methanol (1, 1'), ACN (2, 2').

Figure 2.
¹H NMR spectra of **1** in: a) CDCl₃ and b) DMSO-*d*₆ with methanol-*d*₃.

Figure 3.

Spectral dependences of the fluorescence quantum yield, $\Phi(\lambda)$ ($\circ$, $\bullet$), excitation anisotropy and normalized absorption spectra of **1**. a) $\Phi(\lambda)$ in hexane (1) and ACN (2); normalized absorption in ACN (3). b) $\Phi(\lambda)$ (1) and normalized absorption (2) in methanol. c) Excitation anisotropy in pTHF (1), DMSO (2), toluene (3), hexane (4), and ACN (5) under observed wavelengths $\lambda_{obs} = \lambda_{fl}^{max}$; normalized absorption in hexane (6). d) Excitation anisotropy in methanol (1, 2) and glycerol (3, 4) under $\lambda_{obs} = 560$ nm (1, 3) and 420 nm (2, 4); normalized absorption in methanol (6). Experimental errors for Φ values are $\pm (5-8) \%$.

Figure 4.

Spectral properties of **1** in methanol. a) Fluorescence spectra under $\lambda_{exc} = 310$ nm (1), 360 nm (2), and 400 nm (3). b) Excitation spectra under the observed wavelength $\lambda_{obs} = 410$ nm (1), 560 nm (2), and normalized absorption spectrum (3). The sharp peaks are the corresponding first- and second-order scattering lines at λ_{exc} and λ_{obs} . c) Electronic model of the potential surface of the S_0 and S_1 states of **1** in methanol. d) Dependences of the fluorescence intensity on excitation irradiance I_p for the short (1) and long (2) wavelength fluorescence emission bands ($\lambda_{fl}^{max} \approx 405$ and 590 nm, respectively).

Figure 5. 2PA spectra of **1** in hexane (1), methanol (2), and ACN (3). Experimental errors of (1–3) are $\pm 15\%$. Normalized linear absorption (1PA) of **1** in hexane (4).

Figure 6.

Images of COS-7 cells incubated with fluorescence probe **1** (50 μM, 1 h) all taken with 60x, oil immersion objective. a) DIC, 40 ms. b) One-photon fluorescence image, 150 ms (filter cube Ex: 377/50 DM: 409 Em: 525/40). c) 3D reconstruction from overlaid two-photon fluorescence images (Ex: 730; Em. long-pass filter 690 nm) 10 μm grid. d) Two-photon fluorescence image (Ex: 730 nm; Em. long-pass 690 nm).

Scheme 1.
Synthesis of fluorescent probe **1**.

Table 1

Major linear photophysical parameters of **1** in different solvents: absorption λ_{abs}^{max} and fluorescence λ_{fl}^{max} maxima, Stokes shifts, maximum extinction coefficients ϵ^{max} , fluorescence lifetimes τ and quantum yields Φ .

N/N	Hexane	Cyclohex.	Toluene	THF	DCM	DMSO	ACN	Methanol
Δf	10^{-4}	0.005	0.013	0.209	0.217	0.264	0.305	0.309
λ_{abs}^{max} , nm	411±1	413±1	416±1	412±1	414±1	412±1	408±1	407±1
λ_{fl}^{max} , nm	488±1	489±1	504±1	566±2	587±2	598±2	634±2	591±2
Stokes shift, nm	77±1	76±1	88±1	154±1	173±1	186±1	226±1	184±1
$\epsilon^{max} \cdot 10^{-3}, M^{-1} \cdot cm^{-1}$	59±3	64±3	59±3	61±3	63±3	60±3	63±3	66±3
Φ	1±0.05	1±0.05	1±0.05	1±0.05	1±0.05	0.85±0.05	0.58±0.05	0.14±0.05
τ , ns	1.27±0.1	1.25±0.1	1.31±0.1	2.0±0.1	2.3±0.1	2.2±0.1	2.2±0.1	1.5±0.1 0.3±0.1