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Facile Suzuki Coupling Strategy toward New Nile Red Derivatives with Improved Two-Photon Brightness

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Chemical fine-tuning of fluorophores is a pivotal step towards development of next generation fluorescent dyes for microscopy. With the advent of high-resolution two-photon excitation fluorescence imaging, there is a growing demand for very sensitive laser dyes that can be efficiently excited using commercial Ti:sapphire laser sources in the first near-infrared window (NIR-I, 780–1020 nm). Using the fluorescent dye Nile Red as the lead structure, we report a robust and concise Suzuki coupling approach for the synthesis of 14 new Nile Red analogues that feature extended π ring systems and diverse functionalities. For this set, we gauged their two-photon excitation efficiency in NIR-I as well as evaluated their general fluorescent properties (emission wavelength, Stokes shift, quantum yield and solvatochromism). Several of the new fluorophores were found to display very favorable characteristics. In particular, the derivative featuring a 4-aminophenyl group in the 2-position of Nile Red exhibited extreme solvent sensitivity, and the thien-2-yl substituted Nile Red derivative showed significantly redshifted emission, large Stokes shift and high two-photon brightness.

Introduction

As specialized applications of fluorescence microscopy are becoming increasingly prevalent, there is a growing demand for tailored fluorescent probes.[1–3] Such fluorescent probes should, for example, be able to convey detailed information about biological processes in real-time in live tissue. Usually, the emissive output is non-invasively monitored by, for example, two-photon excitation microscopy (2PEM),[4] laser-scanning microscopy (LSM) or fluorescence-lifetime imaging microscopy (FLIM).[1,5–8] To observe biomolecular interactions at the nanoscale, Förster resonance energy transfer (FRET) microscopy, or super-resolution microscopy can be employed.[8,9]

Fluorescence microscopy on living cells necessitates fluorescent dyes that are highly emissive and that absorb at low energies in order to diminish tissue damage (phototoxicity)[10,11] and photobleaching.[10] However, most commercially available emissive dyes are not useful for live cell imaging due to poor photostability, solubility and/or two-photon properties.[12–14] In addition, they may suffer from signal attenuation as a result of aggregation-caused quenching, or inner filter effects due to low Stokes shifts.[13,15] One of today's preferred fluorescent dyes for live cell imaging is Nile Red (NR), which exhibits many favorable properties, including significant solvatochromism and low background signal due to very low fluorescence in water,[16,17] as well as moderate two-photon excitation fluorescence (2PEF).[16] Moreover, it is well known that Nile Red has a large Stokes shift compared to many other organic dyes.[17,18] Nevertheless, Nile Red has never been chemically optimized for live cell imaging, and very little work has been done to further tweak its photophysical properties for example 2PEF microscopy.[16,17,19,20] This leaves a vacuum of opportunities for developing new analogs using simple, inexpensive methods. In a recent paper, we explored the synthetic opportunities and presented a series of Nile Red analogues with small substituents in various positions.[19] In this work, we deploy a straightforward divergent synthesis for preparing a library of novel Nile Red analogues that are assessed for their fluorescent potential.

The properties of fluorescence dyes are heavily affected by changes in their chemical structure.[4] A more extended conjugated π-system usually results in a redshifted fluorescence compared to smaller similar aromatic systems that have fluorescent emission at higher energies and lower wavelengths.[21] Moreover, the fluorescence can be affected by introducing new functional groups or heteroatoms. Heavy atoms like Br or I are known to have a negative effect due to fluorescent quenching of dyes.[21] Heteroatoms may augment solvent sensitivity by enabling hydrogen bonds to protic solvents. Furthermore, a heteroatom in an aromatic π-system may take part in the system and lower the energy between the ground and excited states of the dye giving rise to redshifted

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emission.[21,22] The emission of a dye can as well be affected by the introduction of electron withdrawing or donating groups, and are often causing a broadening of the spectral maxima.[21,22] Furthermore, the addition of acid or base functional groups gives rise to a pH-dependency.[21,23] Introduction of both an electron donating and a withdrawing group, may enhance the dipole moment and increase the Stokes shift, solvent sensitivity,[21,24] and the two-photon absorptivity.[25]

In this work, we have added different substituents to the 2- and 3-positions of NR, to manipulate the exiting pull-push system that arise from the 9-diethylamino group and the 5-oxo group. This pull-push dipole system is what underlies its large Stokes shift compared to other known dyes such as BODIPY, perylene, rhodamines, etc. The large Stokes shift lowers the risk of the inner filter effects that arise when the dye concentration is too high.[12]

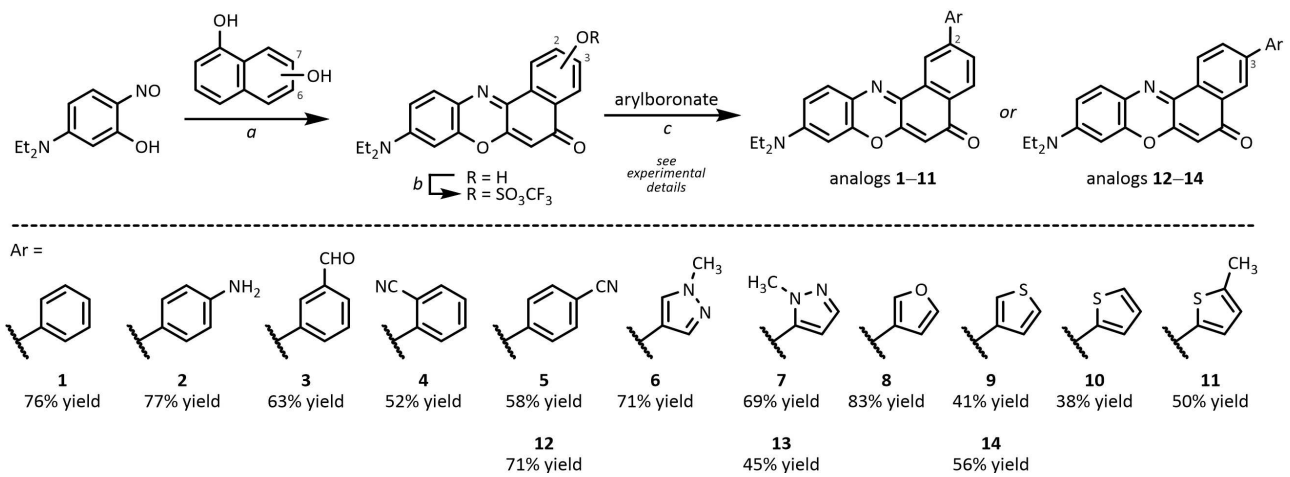
Results and Discussion

We report the synthesis of new NR analogues to probe how various (hetero)aryl substituents influence the fluorescent properties of NR, and to examine the new analogues' eligibility for Ti:sapphire-based microscopy cell imaging. Our approach was to employ the known 2- and 3-hydroxy substituted NR as starting materials for further functionalization (Scheme 1). Specifically, the hydroxy NR can be converted into the corresponding triflate derivatives[19,26] to facilitate functionalization of NR with different aromatic and heterocyclic moieties to obtain a library of compounds. For the introduction of the substituents, the triflate group function as a leaving group in Suzuki cross-couplings by palladium-catalyzed chemistry to form NR analogues **1**–**14**. The triflation takes place using *N*-phenyl-bis(trifluoromethanesulfonimide) with triethylamine as base in various organic solvents.[19,26] Alternatively, trifluoromethanesulfonic anhydride was found to increase the reaction rate but also produced vast amounts of biproducts. Therefore, *N*-phenyl-bis(trifluoromethanesulfonimide) is our preferred choice of triflation agent to form the triflates in yields of 70–77 %. For the Suzuki cross-couplings, $Pd_2(dba)_3$ was used as the catalyst in combination with 2-dicyclohexylphosphino-2′,6′-dimethoxybiphenyl (SPhos) as ligand and sodium carbonate as base, which gave the new NR analogues **1**–**14** in yields of 38–83 %. The overall synthetic route from the starting 3-(diethylamino)phenol to the novel analogues of NR is shown in Scheme 1.

With the synthesized NR analogues in hand, their photophysical properties were examined. The wavelengths of maximum absorption (λ_{max}^{1PA}) and emission (λ_{max}^{em}), Stokes shift ($\Delta\lambda$), fluorescence quantum yield (Φ), molar extinction coefficient (ε), brightness ($\Phi\varepsilon$), two-photon absorption (2PA) and two-photon brightness ($\Phi\sigma_2$) are summarized in Table 1. All properties were determined in three different solvents (methanol, chloroform and toluene) to assess the analogues' degree of solvatochromism.

From Table 1 a clear tendency of a redshift is observed for all the new analogues due to the added substituent, which enlarges the conjugated π-system. In the case of analogues **1**–**14** it is hard to point out a trend of the red-shifting from the added functional groups. Furthermore, the emission does not appear to be notably position-dependent (Figure 1). The Stokes shift of the new fluorophores are improved in toluene and for the most part in chloroform as well. However, most of the new analogues are inferior to NR in MeOH in terms of the Stokes shift. Of all the molecules, NR shows the largest solvatochromic effect (Figure 1A), but the new analogues are still reasonably solvatochromic and the Stokes shifts are still large compared to other classes of organic fluorophores.[27]

In general, addition of the new functional groups onto the 2- and 3-positions have shown to lower the efficiency of the dye. This is shown by the lower QY for all new analogues (Φ, Table 1). All compounds follow the same trend of being darker going from toluene to methanol. For compound **2** with a 4-aminophenyl substitution it is striking how dependent the fluorescence is on the media: it has a QY close to 0 in methanol and chloroform but a decent QY of 0.40 in toluene. This



Scheme 1. *Reagents and conditions:* (*a,b*) see Ref. [19, 26], (*c*) arylboronate, 4 mol % $Pd_2(dba)_3$, 8 mol % SPhos, 2 M Na_2CO_3, ethanol/toluene, 100 °C.

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Table 1. Photophysical properties of NR and the analogs **1**–**14**.

#	Substituent on Nile Red	Solvent	λ_{max}^{1PA}[nm]	λ_{max}^{em}[nm]	Stokes shift Δλ	Φ	ε[mM^{-1} cm^{-1}]	Φε[mM^{-1} cm^{-1}]	λ_{max}^{2PA}[nm]	σ_2[GM]	$\Phi\sigma_2$ [GM]
NR	H	Toluene	521	566	45	0.83	29.9	24.8	875	44	36.5
		Chloroform	537	595	58	0.64	44.3	28.4	875	50.5	32.3
		Methanol	550	636	86	0.34	42.8	14.5	873	20.3	6.9
1	2-phenyl	Toluene	527	583	46	0.53	8.7	4.5	873	20.9	11.1
		Chloroform	552	610	58	0.43	10.7	4.6	874	31.4	13.5
		Methanol	555	641	86	0.24	10	2.4	873	13.3	3.2
2	2-(*p*-amino-phenyl)	Toluene	526	578	52	0.4	29.3	11.7	873	19	7.6
		Chloroform	542	607	65	0.07	28.3	2	876	21.4	1.5
		Methanol	555	641	86	<0.01	29	0.5	876	<0.01	<0.01
3	2-(*m*-formyl-phenyl)	Toluene	532	583	51	0.54	29.8	16.1	872	75.6	40.8
		Chloroform	548	610	62	0.49	39.5	19.4	874	105.7	51.8
		Methanol	558	641	83	0.25	25.6	6.4	872	44.8	11.2
4	2-(*o*-cyano-phenyl)	Toluene	530	583	53	0.53	32.1	17	873	79.1	41.9
		Chloroform	548	609	61	0.51	45.7	23.3	873	90.8	46.3
		Methanol	560	641	81	0.27	32.5	8.8	871	43.7	11.8
5	2-(*p*-cyano-phenyl)	Toluene	536	586	50	0.55	32.7	18	870	69.1	38
		Chloroform	552	612	60	0.48	42.9	20.6	873	106.5	51.1
		Methanol	561	643	82	0.26	36.3	9.4	871	42.6	11.1
6	2-(1-methyl-pyrazol-4-yl)	Toluene	526	577	51	0.56	29.8	16.7	873	106.8	59.8
		Chloroform	543	607	64	0.48	24.2	11.6	876	117.5	56.4
		Methanol	555	641	86	0.27	25.4	6.9	876	53.3	14.4
7	2-(1-methyl-pyrazol-5-yl)	Toluene	533	583	50	0.61	37.4	22.8	870	80.2	48.9
		Chloroform	550	610	60	0.49	36.6	17.9	873	107.3	52.6
		Methanol	561	642	81	0.26	36.6	9.5	871	46.5	12.1
8	2-(furan-3-yl)	Toluene	527	578	51	0.52	22.2	11.5	872	63.7	33.1
		Chloroform	544	607	63	0.46	21	9.7	875	87.2	40.1
		Methanol	555	640	85	0.25	21.5	5.4	874	40	10
9	2-(thien-3-yl)	Toluene	528	579	51	0.51	21.7	11.1	871	68	34.7
		Chloroform	545	608	63	0.47	25.1	11.8	875	97	45.6
		Methanol	556	642	86	0.25	33.9	8.5	875	45.6	11.4
10	2-(thien-2-yl)	Toluene	531	581	50	0.51	32.1	16.4	872	133.1	67.9
		Chloroform	548	610	62	0.47	36.4	17.1	876	184.9	86.9
		Methanol	560	643	83	0.26	33.8	8.8	876	65	16.9
11	2-(5-methyl-thien-2-yl)	Toluene	531	580	49	0.53	28.6	15.2	873	143.6	76.1
		Chloroform	547	607	60	0.44	28.7	12.6	877	170	74.8
		Methanol	561	643	82	0.12	36.7	4.4	878	84.2	10.1
12	3-(*p*-cyano-phenyl)	Toluene	540	589	49	0.52	37.1	19.3	878	63.5	33
		Chloroform	555	614	59	0.47	40.6	19.1	876	62.5	29.4
		Methanol	564	642	78	0.3	34.3	10.3	873	26.3	7.9
13	3-(1-methyl-pyrazol-5-yl)	Toluene	535	586	51	0.54	35.5	19.2	877	61.5	33.2
		Chloroform	552	610	58	0.47	41.7	19.6	876	64.7	30.4
		Methanol	562	641	79	0.25	33	8.3	872	35.2	8.8
14	3-(thien-3-yl)	Toluene	531	581	50	0.56	18.2	10.2	870	38	21.3
		Chloroform	548	611	63	0.5	38	19	875	83.2	41.6
		Methanol	557	644	87	0.27	13.9	3.8	870	27.4	7.4

characteristic is by itself interesting making it a great performer regarding a solvent sensitive turn-on/off probe biomarker. The low quantum yields are in accordance with the compounds' rapid fluorescence decay in methanol and chloroform (<1 ns), suggestive of non-radiative relaxation of the molecule. In toluene, the fluorescence lifetime is 3.0 ns (Figure S44), which is only slightly lower than the other fluorophores (3.8–4.2 ns, data not shown).

For the most part, the new probes are inferior to NR in terms of one-photon microscopy (Figure 1B). In two-photon microscopy, where the maxima of absorption occur at 870–880 nm, the picture is rather different. Looking at two-photon brightness, several of the new compounds are far superior to the original NR (Figure 1B). The most promising probes are the thiophen-2-yl substituted compounds **10** and **11**, which are twice as bright compared to NR (Figure 2). In two-photon studies, there is also a noticeable difference associated with the position of the heteroatom in the substituent and furthermore, derivatives substituted in the 2-position are brighter than the 3-positioned counterparts. Other qualified candidates among the new NR analogues are compounds **6** and **7** substituted in the 2-position with methylated pyrazoles having useful one-photon and potent two-photon characteristics (Table 1, Figure 2).

Conclusions

In conclusion, we have synthesized a range of new NR analogues by a simple Suzuki-Miyaura cross-coupling divergent synthetic method using triflate-substituted NR as starting materials. We have increased the π-system of the benzophenoxazinone structure by addition of heterocyclic groups in the form of different thiophenes **9**–**11**, **14** and furan **8**. The thiophene substituents differed from the furan substituents in inciting a

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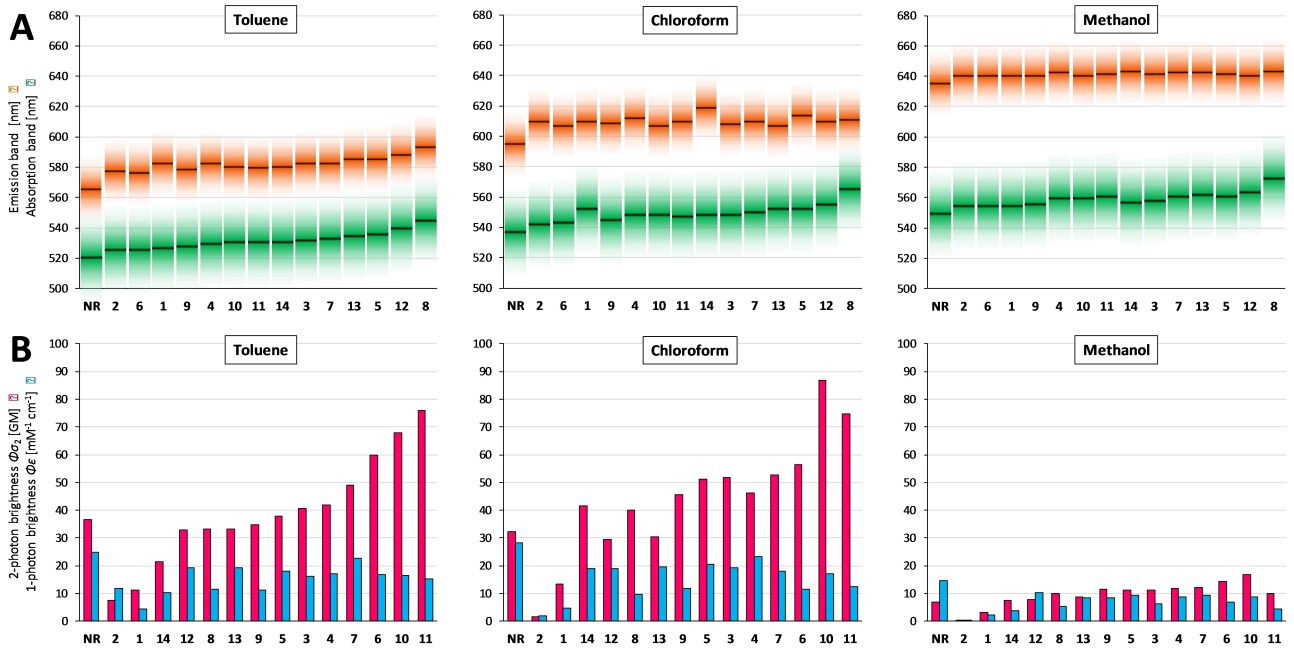

Figure 1. (A) The maxima of the absorption and emission bands of **NR** and compounds **1**–**14** in toluene (least polar, $\varepsilon = 2.4$), chloroform ($\varepsilon = 4.8$) and methanol (most polar, $\varepsilon = 33$) arranged in order of increasing peak absorption wavelength in toluene. (B) 1-Photon brightness ($\Phi\varepsilon$) and 2-photon brightness ($\Phi\sigma_2$) of **NR** and compounds **1**–**14**. The analogues are arranged in order of increasing two-photon brightness in toluene.

more prominent Stokes shift (i.e., 71 nm for furan-3-yl vs 86 nm for the corresponding thien-3-yl in methanol). In addition, analogues **10** and **11** showed the highest brightness in 2PEF indicating that thiophen-2-yl substituents have a pronounced impact on the photophysical properties of NR by increasing Stokes shift and the two-photon brightness, especially in non-polar solvents. From the one-photon spectroscopic studies we learned that the position of substitution did not have a significant impact on the optical properties. However, regarding 2PEF, the 2-substituted NR analogues have an increased two-photon brightness compared to the 3-substituted equivalents. In conclusion, analogues **10** and **11** appear promising in 2PEF studies and analogue **2** exhibit strong solvent dependency. Moreover, analogues **6** and **7** possess elevated photophysical traits in general, which make them promising new fluorescent dyes for a wide range of applications.

Experimental Section

General: All chemicals and solvents were used as obtained from Sigma-Aldrich and Fluorochem. TLC was carried out on precoated Kieselgel 60 F_{254} aluminum plates. Column chromatography was performed using Merck Kieselgel 60 (0.040–0.063 mm). NMR spectra were recorded on a Bruker AVANCE III 400 MHz instrument. ^{1}H NMR was recorded at 400 MHz and ^{13}C NMR at 101 MHz. Chemical shifts are reported in ppm. All NMR spectra were recorded using either chloroform-d (solvent residual peak at 7.26 ppm in ^{1}H NMR and 77.16 ppm in ^{13}C NMR) or DMSO-d_6 (solvent residual peak at 2.50 ppm in ^{1}H NMR and 39.52 ppm in ^{13}C NMR). Electrospray ionization (ESI) high-resolution mass spectra (HRMS) was recorded on a Bruker Daltonics-micrOTOF QII ESI Qq-TOF mass spectrometer. All recorded NMR spectra are found in the Supporting Information. Absorption spectra were recorded on a Perkin Elmer Lambda 35 UV/Vis Spectrometer from 350 to 800 nm. Emission spectra were recorded on a Perkin Elmer LS 55 Luminescence Spectrometer in the range from 500 to 800 nm. Two photon absorption measurements were made using a custom built multiphoton excitation spectrofluorometer. The excitation source used was a Ti:Sa laser (HPeMaiTai DeepSee, Spectra Physics, Mountain View, CA). The laser was sent to the sample trough a CFI S Plan Fluor ELWD 60X objective (Nikon). The laser power was monitored by a power meter (PM100D with a S142 C head, Thorlabs Sweden AB Goteborg, Sweden). The two photon excited emission was collected and passed through a Multiphoton-Emitter HC 680/SP (AHF analysentechnik AG, Tuebingen, Germany) to a multimode optical fiber (M200 L02S- A, Thorlabs Sweden AB Goteborg, Sweden). The emitted light was then sent to a monochromator (ARC-SP2155, BFi OPTiLAS, Sweden) and the spectra was recorded using a cooled CCD camera (PIXIS 400B, Princeton Instruments - New Jersey, USA). A motorized XY stage (Nikon) was used as a sample holder. The laser, laser power, camera and XY stage was controlled using ImageJ72 and custom scripts. The absolute 2-photon absorption cross sections were calculated using a relative fluorescence intensity technique as described previously.[28,29] The 2PA spectra were recorded in the range from 780 to 1020 nm using samples at 20 μM concentration, except compound **2**, which was recorded at 500 μM concentration in chloroform and methanol. Nile Red in the respective solvents served as references having known literature values for 2-photon cross-section.[20] QYs were measured at room temperature using the relative method.[30] Rhodamine B in EtOH (F 0.50)[31] and Nile Red in the respective solvents (F 0.34 in MeOH, F 0.64 in $CHCl_3$, and F 0.83 in toluene) were used as standards. Samples in MeOH, $CHCl_3$, and toluene were excited at 558, 546, and 526 nm, respectively.

General procedure for the Suzuki-Miyaura cross-couplings. To a mixture of toluene and EtOH (50 mL, 4:1) was added triflated Nile Red[19,26] (1.00 mmol), boronic acid derivative (2.00 mmol), SPhos (80 μmol, 8 mol%), $Pd_2(dba)_3$ (40 μmol, 4 mol%) and 2 M aq.

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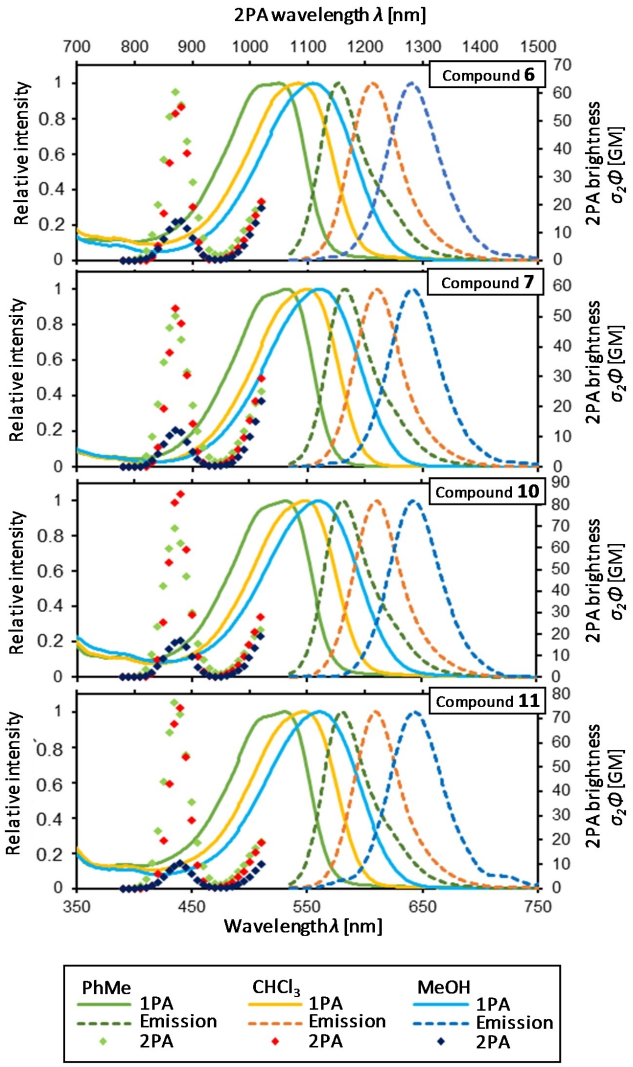

Figure 2. 1PA spectra (350–760 nm, solid lines), emission spectra (520–750 nm, dashed lines), and 2PA spectra (780–1020 nm, squares) of compounds **6**, **7**, **10** and **11** in methanol (blue), chloroform (yellow) and toluene (green). 1PA and emission spectra are represented in relative intensity (left vertical axes), whereas 2PA spectra are represented as 2PA brightness ($\sigma_2\Phi$) in GM units (right vertical axes) in 10 nm increments (top horizontal axis).

Na_2CO_3 (15 mL). The reaction mixture was flushed with N_2 and stirred at 100 °C overnight. The reaction mixture was concentrated under reduced pressure and the residue was portioned between EtOAc (250 mL) and water (200 mL). The aqueous layer was extracted with EtOAc (50 mL) and the organic phase was washed with water (2×100 mL). The solvent was evaporated and the crude solid was purified by column chromatography (EtOAc:PE 0–100 %).

9-(Diethylamino)-2-phenyl-5*H*-benzo[*a*]phenoxazin-5-one (1). The general procedure was applied with 2-triflated Nile Red (42 mg, 90 µmol) and phenylboronic acid (35 mg, 0.18 mmol) to obtain compound **1** (27 mg, 68 µmol). Yield: 76 %. ^{1}H NMR ($CDCl_3$): δ 8.85 (d, J=1.8 Hz, 1H, H-1), 8.34 (d, J=8.2 Hz, 1H, H-4), 7.86 (dd, J=8.2, 1.8 Hz, 1H, H-3), 7.77 (m, 2H, phenyl H-2, phenyl H-6), 7.60 (d, J=9.1 Hz, 1H, H-11), 7.51 (m, 2H, phenyl H-3, phenyl H-5), 7.42 (m, 1H, phenyl H-4), 6.64 (dd, J=9.1, 2.8 Hz, 1H, H-10), 6.44 (d, J=2.8 Hz, 1H, H-8), 6.37 (s, 1H, H-6), 3.44 (q, J=7.1 Hz, 4H, CH_2), 1.25 (t, J=7.1 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$): δ 183.5 (C-5), 152.3 (C-6a), 150.8 (C-9), 146.8 (C-7a), 144.0 (C-12a), 140.3 (C-2), 139.9 (phenyl C-1), 132.5 (C-12b), 131.2 (C-11), 130.6 (phenyl C-4), 128.9 (phenyl C-3, phenyl C-5), 128.6 (C-3), 128.1 (C-4a), 127.5 (phenyl C-2, phenyl C-6), 126.4 (C-4), 125.0 (C-11a), 122.2 (C-1), 109.8 (C-10), 105.8 (C-6), 96.3 (C-8), 45.1 (CH_2), 12.6 (CH_3). HRMS (ESI): calculated for $C_{26}H_{23}N_2O_2$ [M+H$^+$]: 395.1754; found: 395.1746.

2-(4-Aminophenyl)-9-(diethylamino)-5*H*-benzo[*a*]phenoxazin-5-one[32] **(2)**. The general procedure was applied with 2-triflated Nile Red (138 mg, 0.30 mmol) and 4-aminobenzeneboronic acid pinacol ester (131 mg, 0.60 mmol) to obtain compound **2** (93 mg, 0.23 mmol). Yield: 77 %. ^{1}H NMR ($CDCl_3$): δ 8.78 (d, J=1.9 Hz, 1H, H-1), 8.28 (d, J=8.3 Hz, 1H, H-4), 7.80 (dd, J=8.3, 1.9 Hz, 1H, H-3), 7.65–7.55 (m, 3H, H-11, aniline H-2, aniline H-6), 6.80 (d, J=8.5 Hz, 2H, aniline H-3, aniline H-5), 6.62 (dd, J=9.1, 2.7 Hz, 1H, H-10), 6.41 (d, J=2.7 Hz, 1H, H-8), 6.34 (s, 1H, H-6), 3.42 (q, J=7.1 Hz, 4H, CH_2), 1.23 (t, J=7.1 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$): δ 183.7 (C-5), 152.2 (C-6a), 150.7 (C-9), 146.8 (C-7a), 146.7 (aniline C-4), 144.0 (C-2), 140.2 (C-12a), 132.4 (C-12b), 131.1 (C-11), 130.3 (aniline C-1), 129.7 (C-4a), 128.4 (aniline C-2, aniline C-6), 127.8 (C-3), 126.3 (C-4), 125.0 (C-11a), 120.9 (C-1), 115.4 (aniline C-3, aniline C-5), 109.7 (C-10), 105.7 (C-6), 96.3 (C-8), 45.1 (CH_2), 12.6 (CH_3). HRMS (ESI): calculated for $C_{26}H_{24}N_3O_2$ [M+H$^+$]: 410.1863; found: 410.1843.

9-(Diethylamino)-2-(3-formylphenyl)-5*H*-benzo[*a*]phenoxazin-5-one (3): The general procedure was applied with 2-triflated Nile Red (50 mg, 0.11 mmol) and 5-methylthiophene-2-boronic acid pinacol ester (36 mg, 0.24 mmol) to obtain compound **3** (29 mg, 69 µmol). Yield: 63 %. ^{1}H NMR ($CDCl_3$): δ 10.15 (s, 1H, CHO), 8.89 (d, J=1.9 Hz, 1H, H-1), 8.39 (d, J=8.2 Hz, 1H, H-4), 8.28 (t, J=1.9 Hz, 1H, benzaldehyde H-2), 8.04 (ddd, J=7.7, 1.9, 1.3 Hz, 1H, benzaldehyde H-4), 7.93 (dt, J=7.7, 1.3 Hz, 1H, benzaldehyde H-6), 7.89 (dd, J=8.2, 1.9 Hz, 1H, H-3), 7.68 (t, J=7.7 Hz, 1H, benzaldehyde H-5), 7.65 (d, J=9.1 Hz, 1H, H-11), 6.68 (dd, J=9.1, 2.7 Hz, 1H, H-10), 6.47 (d, J=2.7 Hz, 1H, H-8), 6.40 (s, 1H, H-6), 3.48 (q, J=7.1 Hz, 4H, CH_2), 1.27 (t, J=7.1 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$): δ 192.2 (CHO), 183.3 (C-5), 152.4 (C-6a), 151.0 (C-9), 146.9 (C-7a), 142.4 (C-2), 141.3 (benzaldehyde C-1), 139.7 (C-12a), 137.1 (benzaldehyde C-3), 133.4 (benzaldehyde C-6), 132.6 (C-12b), 131.3 (C-11), 131.1 (C-4a), 129.7 (benzaldehyde C-4), 129.4 (benzaldehyde C-2), 128.49 (benzaldehyde C-5), 128.46 (C-3), 126.7 (C-4), 125.1 (C-11a), 122.3 (C-1), 109.9 (C-10), 105.9 (C-6), 96.3 (C-8), 45.1 (CH_2), 12.6 (CH_3). HRMS (ESI): calculated for $C_{27}H_{23}N_2O_3$ [M+H$^+$]: 423.1703; found: 423.1704.

9-(Diethylamino)-2-(2-cyanophenyl)-5*H*-benzo[*a*]phenoxazine-5-one (4). The general procedure was applied with 2-triflated Nile Red (50 mg, 0.11 mmol) and 2-cyanophenylboronic acid (35 mg, 0.24 mmol) to obtain compound **4** (24 mg, 57 µmol). Yield: 52 %. ^{1}H NMR ($CDCl_3$): δ 8.81 (d, J=1.8 Hz, 1H, H-1), 8.41 (d, J=8.2 Hz, 1H, H-4), 7.82 (m, 2H, H-3, phenyl H-6), 7.68 (m, 2H, phenyl H-3, phenyl H-4), 7.57 (d, J=9.1 Hz, 1H, H-11), 7.51 (td, J=7.5, 1.4 Hz, 1H, phenyl H-5), 6.64 (dd, J=9.1, 2.7 Hz, 1H, H-10), 6.44 (d, J=2.7 Hz, 1H, H-8), 6.39 (s, 1H, H-6), 3.44 (q, J=7.1 Hz, 4H, CH_2), 1.25 (t, J=7.1 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$): δ 183.1 (C-5), 152.4 (C-6a), 151.0 (C-9), 146.9 (C-7a), 144.8 (phenyl C-1), 141.0 (C-12a), 139.4 (C-2), 133.8 (phenyl C-4), 132.9 (phenyl C-3), 132.4 (C-12b), 131.5 (C-4a), 131.3 (phenyl C-6), 130.3 (C-11), 130.0 (C-3), 128.2 (phenyl C-5), 126.3 (C-4), 125.1 (C-11a), 124.3 (C-1), 118.4 (*C*N), 111.6 (phenyl C-2), 109.9 (C-10), 105.9 (C-6), 96.3 (C-8), 45.1 (CH_2), 12.6 (CH_3). HRMS (ESI): calculated for $C_{27}H_{22}N_3O_2$ [M+H$^+$]: 420.1707; found: 420.1715.

9-(Diethylamino)-2-(4-cyanophenyl)-5*H*-benzo[*a*]phenoxazin-5-one[32] **(5)**. The general procedure was applied with 2-triflated Nile Red (42 mg, 90 µmol) and 4-cyanophenyl boronic acid (26 mg, 0.18 mmol) to obtain compound **5** (22 mg, 52 µmol). Yield: 58 %. ^{1}H NMR ($CDCl_3$): δ 8.84 (d, J=1.8 Hz, 1H, H-1), 8.37 (d, J=8.2 Hz, 1H, H-4), 7.90–7.75 (m, 5H, H-3, phenyl H-2, phenyl H-3, phenyl H-5, phenyl H-6), 7.62 (d, J=9.1 Hz, 1H, H-11), 6.68 (dd, J=9.1, 2.7 Hz,

1H, H-10), 6.47 (d, J = 2.7 Hz, 1H, H-8), 6.39 (s, 1H, H-6), 3.48 (q, J = 7.1 Hz, 4H, CH_2), 1.28 (t, J = 7.1 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$): δ 183.0 (C-5), 152.4 (C-6a), 151.1 (C-9), 146.9 (C-7a), 144.8 (phenyl C-1), 141.7 (C-2), 139.4 (C-12a), 132.7 (phenyl C-2, phenyl C-6), 132.6 (C-12b), 131.4 (C-1), 131.3 (C-3), 128.3 (C-11), 128.1 (phenyl C-3, phenyl C-5), 126.8 (C-4a), 125.0 (C-11a), 122.5 (C-4), 118.8 (*C*N), 111.7 (phenyl C-4), 110.0 (C-10), 105.9 (C-6), 96.3 (C-8), 45.2 (CH_2), 12.6 (CH_3). HRMS (ESI): calculated for $C_{27}H_{22}N_3O_2$ [M + H$^+$]: 420.1707; found: 420.1703.

9-(Diethylamino)-2-(1-methyl-1*H*-pyrazol-4-yl)-5*H*-benzo[*a*]-phenoxazin-5-one (6). The general procedure was applied with 2-triflated Nile Red (50 mg, 0.11 mmol) and 1-methylpyrazole-4-boronic acid pinacol ester (50 mg, 0.24 mmol) to obtain compound **6** (31 mg, 78 μmol). Yield: 71%; ^{1}H NMR ($CDCl_3$): δ 8.67 (d, J = 2.0 Hz, 1H, H-1), 8.26 (d, J = 8.0 Hz, 1H, H-4), 7.96 (d, J = 1.0 Hz, 1H, pyrazole H-3), 7.83 (d, J = 1.0 Hz, 1H, pyrazole H-5), 7.71 (dd, J = 8.0, 2.0 Hz, 1H, H-3), 7.61 (d, J = 9.0 Hz, 1H, H-11), 6.65 (dd, J = 9.0, 2.5 Hz, 1H, H-10), 6.44 (d, J = 2.5 Hz, 1H, H-8), 6.33 (s, 1H, H-6), 3.99 (s, 3H, pyrazole CH_3), 3.42 (q, J = 7.0 Hz, 4H, CH_2), 1.23 (t, J = 7.0 Hz, 6H, N($CH_2C\underline{H}_3$)). ^{13}C NMR ($CDCl_3$): δ 183.3 (C-5), 152.1 (C-6a), 150.8 (C-9), 146.8 (C-7a), 137.3 (pyrazole C-3), 135.7 (C-2), 132.6 (C-12b), 131.1 (C-11), 129.8 (C-4a), 127.8 (pyrazole C-5), 126.8 (C-3), 126.5 (C-4), 124.9 (C-11a), 122.6 (pyrazole C-4), 119.8 (C-1), 109.7 (C-10), 105.7 (C-6), 96.3 (C-8), 45.1 (CH_2), 39.2 (pyrazole CH_3), 12.6 (CH_3). The signal for C-12a is missing or overlapping; HRMS (ESI): calculated for $C_{24}H_{23}N_4O_2$ [M + H$^+$]: 399.1816; found: 399.1806.

9-(Diethylamino)-2-(1-methyl-1*H*-pyrazol-5-yl)-5*H*-benzo[*a*]-phenoxazin-5-one (7). The general procedure was applied with 2-triflated Nile Red (327 mg, 0.70 mmol) and 1-methyl-1*H*-pyrazole-5-boronic acid pinacol ester (292 mg, 1.40 mmol) to obtain compound **7** (193 mg, 0.48 mmol). Yield: 69%; ^{1}H NMR ($CDCl_3$): δ 8.73 (d, J = 1.8 Hz, 1H, H-1), 8.38 (d, J = 8.1 Hz, 1H, H-4), 7.69 (dd, J = 8.1, 1.8 Hz, 1H, H-3), 7.61 (d, J = 9.1 Hz, 1H, H-11), 7.58 (d, J = 1.8 Hz, 1H, pyrazole H-3), 6.69 (dd, J = 9.1, 2.8 Hz, 1H, H-10), 6.49 (m, 2H, H-8, pyrazole H-3), 6.42 (s, 1H, H-6), 4.00 (s, 3H, pyrazole CH_3), 3.49 (q, J = 7.1 Hz, 4H, CH_2), 1.28 (t, J = 7.1 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$): δ 183.0 (C-5), 152.4 (C-6a), 151.1 (C-9), 147.0 (C-7a), 142.9 (C-2), 139.3 (C-12a), 138.7 (pyrazole C-3), 133.6 (C-12b), 132.4 (pyrazole C-5), 131.4 (C-11), 131.3 (C-4a), 129.8 (C-3), 126.3 (C-4), 125.1 (C-11a), 123.9 (C-1), 110.0 (C-10), 106.8 (pyrazole C-4), 105.9 (C-6), 96.3 (C-8), 45.2 (CH_2), 37.8 (pyrazole CH_3), 12.6 (CH_3). HRMS (ESI): calculated for $C_{24}H_{23}N_4O_2$ [M + H$^+$]: 399.1816; found: 399.1817.

9-(Diethylamino)-2-(furan-3-yl)-5*H*-benzo[*a*]phenoxazin-5-one (8). The general procedure was applied with 2-triflated Nile Red (50 mg, 0.11 mmol) and furan-3-boronic acid (27 mg, 0.24 mmol) to obtain compound **8** (35 mg, 91 μmol). Yield: 83%. ^{1}H NMR ($CDCl_3$): δ 8.71 (d, J = 1.8 Hz, 1H, H-1), 8.28 (d, J = 8.2 Hz, 1H, H-4), 7.95 (dd, J = 1.5, 1.0 Hz, 1H, furan H-2), 7.74 (dd, J = 8.2, 1.8 Hz, 1H, H-3), 7.62 (d, J = 9.1 Hz, 1H, H-11), 7.54 (dd, J = 2.0, 1.0 Hz,1H, furan H-5), 6.90 (dd, J = 2.0, 1.5 Hz, 1H, furan H-4), 6.66 (dd, J = 9.1, 2.7 Hz, 1H, H-10), 6.45 (d, J = 2.7 Hz, 1H, H-8), 6.36 (s, 1H, H-6), 3.46 (q, J = 7.1 Hz, 4H, CH_2), 1.26 (t, J = 7.1 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$): δ 183.3 (C-5), 152.2 (C-6a), 150.8 (C-9), 146.8 (C-7a), 144.0 (furan C-5), 139.9 (C-12a), 139.8 (furan C-2), 135.5 (C-2), 133.4 (C-4a), 132.6 (C-12b), 131.1 (C-11), 130.4 (C-11), 127.3 (C-3), 126.4 (C-4), 126.0 (furan C-3), 124.9 (C-11a), 120.5 (C-1), 109.7 (C-10), 109.0 (furan C-4), 105.8 (C-6), 96.3 (C-8), 45.1 (CH_2), 12.6 (CH_3). HRMS (ESI): calculated for $C_{24}H_{21}N_2O_3$ [M + H$^+$]: 385.1547; found: 385.1550.

9-(Diethylamino)-2-(thien-3-yl)-5*H*-benzo[*a*]phenoxazin-5-one (9). The general procedure was applied with 2-triflated Nile Red (50 mg, 0.11 mmol) and thiophene-3-boronic acid (31 mg, 0.24 mmol) to obtain compound **9** (18 mg, 45 μmol). Yield: 41%. ^{1}H NMR ($CDCl_3$): δ 8.85 (d, J = 1.8 Hz, 1H, H-1), 8.31 (d, J = 8.2 Hz, 1H, H-4), 7.86 (dd, J = 8.2, 1.8 Hz, 1H, H-3), 7.71 (dd, J = 2.9, 1.3 Hz, 1H, thiophene H-2), 7.63 (d, J = 9.1 Hz, 1H, H-11), 7.60 (dd, J = 5.0, 1.3 Hz, 1H, thiophene H-5), 7.45 (dd, J = 5.0, 2.9 Hz, 1H, thiophene H-4), 6.67 (dd, J = 9.1, 2.7 Hz, 1H, H-10), 6.46 (d, J = 2.7 Hz, 1H, H-8), 6.37 (s, 1H, H-6), 3.47 (q, J = 7.1 Hz, 4H, CH_2), 1.26 (t, J = 7.1 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$): δ 183.4 (C-5), 152.3 (C-6a), 150.8 (C-9), 146.9 (C-7a), 141.5 (thiophene C-3), 138.6 (C-12a), 138.5 (C-2), 132.6 (C-12b), 131.1 (C-11), 130.4 (C-4a), 127.9 (C-3), 126.54, 126.51, 126.46 (C-4, thiophene C-4, thiophene C-5), 125.0 (C-11a), 122.0 (thiophene C-2), 121.3 (C-1), 109.7 (C-10), 105.8 (C-6), 96.4 (C-8), 45.1 (CH_2), 12.6 (CH_3). HRMS (ESI): calculated for $C_{24}H_{21}N_2O_2S$ [M + H$^+$]: 401.1318; found: 401.1320.

9-(Diethylamino)-2-(thien-2-yl)-5*H*-benzo[*a*]phenoxazin-5-one (10). The general procedure was applied with 2-triflated Nile Red (50 mg, 0.11 mmol) and thiophene-2-boronic acid (31 mg, 0.24 mmol) to obtain compound **10** (17 mg, 42 μmol). Yield 38%; ^{1}H NMR ($CDCl_3$): δ 8.83 (d, J = 1.8 Hz, 1H, H-1), 8.27 (d, J = 8.3 Hz, 1H, H-4), 7.84 (dd, J = 8.3, 1.8 Hz, 1H, H-3), 7.61 (d, J = 9.1 Hz, 1H, H-11), 7.56 (dd, J = 3.6, 1.2 Hz, 1H, thiophene H-3), 7.39 (dd, J = 5.1, 1.2 Hz, 1H, thiophene H-5), 7.15 (dd, J = 5.1, 3.6 Hz, 1H, thiophene H-4), 6.64 (dd, J = 9.1, 2.7 Hz, 1H, H-10), 6.43 (d, J = 2.7 Hz, 1H, H-8), 6.35 (s, 1H, H-6), 3.45 (q, J = 7.1 Hz, 4H, CH_2), 1.25 (t, J = 7.1 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$): δ 183.2 (C-5), 152.2 (C-6a), 150.9 (C-9), 146.9 (C-7a), 143.5 (thiophene C-2), 139.7 (C-12a), 137.2 (C-2), 132.6 (C-12b), 131.2 (C-3), 130.5 (C-4a), 128.3 (thiophene C-3), 127.1 (C-4), 126.5 (thiophene C-5), 126.2 (C-1), 125.0 (C-11a), 124.6 (thiophene C-4), 120.5 (C-11), 109.8 (C-10), 105.8 (C-6), 96.3 (C-8), 45.1 (CH_2), 12.6 (CH_3). HRMS (ESI): calculated for $C_{24}H_{21}N_2O_2S$ [M + H$^+$]: 401.1318; found: 401.1322.

9-(Diethylamino)-2-(5-methylthien-2-yl)-5*H*-benzo[*a*]phenoxazin-5-one (11). The general procedure was applied with 2-triflated Nile Red (50 mg, 0.11 mmol) and 5-methylthiophene-2-boronic acid pinacol ester (54 mg, 0.24 mmol) to obtain compound **11** (23 mg, 55 μmol). Yield: 50%. ^{1}H NMR ($CDCl_3$): δ 8.72 (d, J = 1.8 Hz, 1H, H-1), 8.22 (d, J = 8.2 Hz, 1H, H-4), 7.75 (dd, J = 8.2, 1.8 Hz, 1H, H-3), 7.57 (d, J = 9.1 Hz, 1H, H-11), 7.35 (d, J = 3.5 Hz, 1H thiophene H-3), 6.81–6.76 (m, 1H, thiophene H-4) 6.61 (dd, J = 9.1, 2.7 Hz, 1H, H-10), 6.39 (d, J = 2.7 Hz, 1H, H-8), 6.31 (s, 1H, H-6), 3.42 (q, J = 7.1 Hz, 4H, CH_2), 2.54 (d, J = 1.1 Hz, 3H, thiophene CH_3), 1.24 (t, J = 7.1 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$): δ 183.2 (C-5), 152.2 (C-6a), 150.8 (C-9), 146.8 (C-7a), 141.1 (thiophene C-2), 141.0 (C-12a), 139.8 (thiophene C-5), 137.4 (C-2), 132.6 (C-12b), 131.2 (C-11), 130.1 (C-4a), 126.6 (C-3), 126.5 (thiophene C-4), 126.4 (C-4), 125.0 (C-11a), 124.6 (thiophene C-3), 119.9 (C-1), 109.7 (C-10), 105.7 (C-6), 96.3 (C-8), 45.1 (CH_2), 15.6 (thiophene CH_3), 12.7 (CH_3). HRMS (ESI): calculated for $C_{25}H_{23}N_2O_2S$ [M + H$^+$]: 415.1475; found: 415.1477.

9-(Diethylamino)-3-(4-cyanophenyl)-5*H*-benzo[*a*]phenoxazin-5-one[32] (12). The general procedure was applied with 3-triflated Nile Red (42 mg, 90 μmol) and 4-cyanophenylboronic acid (26 mg, 0.18 mmol) to obtain compound **12** (27 mg, 64 μmol). Yield: 71%. ^{1}H NMR ($CDCl_3$): δ 8.68 (d, J = 8.3 Hz, 1H, H-1), 8.51 (d, J = 2.1 Hz, 1H, H-4), 7.90 (dd, J = 8.3, 2.1 Hz, 1H, H-2), 7.86–7.75 (m, 4H, phenyl H-2, phenyl H-3, phenyl H-5, phenyl H-6), 7.59 (d, J = 9.1 Hz, 1H, H-11), 6.68 (dd, J = 9.1, 2.7 Hz, 1H, H-10), 6.46 (d, J = 2.7 Hz, 1H, H-8), 6.39 (s, 1H, H-6), 3.47 (q, J = 7.1 Hz, 4H, CH_2), 1.27 (t, J = 7.1 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$): δ 182.2 (C-5), 152.4 (C-6a), 151.1 (C-9), 146.9 (C-7a), 144.4 (phenyl C-1), 140.0 (C-3), 139.1 (C-12a), 132.7 (phenyl C-2, phenyl C-6), 132.2 (C-12b), 132.0 (C-4a), 131.3 (C-11), 129.5 (C-2), 127.8 (phenyl C-3, phenyl C-5), 125.3 (C-11a), 124.8 (C-1), 124.3 (C-4), 118.8 (CN), 111.5 (phenyl C-4), 110.0 (C-10), 105.8 (C-6), 96.3 (C-8), 45.2 (CH_2), 12.6 (CH_3). HRMS (ESI): calculated for $C_{27}H_{22}N_3O_2$ [M + H$^+$]: 420.1707; found: 420.1701.

9-(Diethylamino)-3-(1-methyl-1*H*-pyrazol-5-yl)-5*H*-benzo[*a*]-phenoxazin-5-one (13). The general procedure was applied with 3-triflated Nile Red (50 mg, 0.11 mmol) and 1-methyl-1*H*-pyrazole-5-

boronic acid pinacol ester (50 mg, 0.24 mmol) to obtain compound **13** (20 mg, 50 μmol). Yield: 45%. ^{1}H NMR ($CDCl_3$): δ 8.71 (d, J = 8.3 Hz, 1H, H-1), 8.38 (d, J = 1.9 Hz, 1H, H-4), 7.76 (dd, J = 8.3, 1.9 Hz, 1H, H-2), 7.61 (d, J = 9.1 Hz, 1H, H-11), 7.56 (d, J = 1.9 Hz, 1H, pyrazole H-3), 6.69 (dd, J = 9.1, 2.7 Hz, 1H, H-10), 6.48 (d, J = 2.7 Hz, 1H, H-8), 6.46 (d, J = 1.9 Hz, 1H, pyrazole H-4), 6.41 (s, 1H, H-6), 4.00 (s, 3H, pyrazole CH_3), 3.48 (q, J = 7.1 Hz, 4H, CH_2), 1.27 (t, J = 7.1 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$): δ 183.1 (C-5), 152.4 (C-6a), 151.0 (C-9), 146.9 (C-7a), 142.7 (C-3), 139.1 (C-12a), 138.7 (pyrazole C-3), 132.0 (C-12b), 131.9 (pyrazole C-5), 131.7 (C-4a), 131.3 (C-11), 131.1 (C-2), 125.5 (C-4), 125.2 (C-11a), 124.4 (C-1), 110.0 (C-10), 106.7 (pyrazole C-4), 105.8 (C-6), 96.3 (C-8), 45.2 (CH_2), 37.9 (pyrazole CH_3), 12.6 (CH_3). HRMS (ESI): calculated for $C_{24}H_{23}N_4O_2$ [M+H$^+$]: 399.1816; found: 399.1810.

9-(Diethylamino)-3-(thien-3-yl)-5*H*-benzo[*a*]phenoxazin-5-one (14). The general procedure was applied with 3-triflated Nile Red (50 mg, 0.11 mmol) and thiophene-3-boronic acid (31 mg, 0.24 mmol) to obtain compound **14** (25 mg, 62 μmol). Yield: 56%. ^{1}H NMR ($CDCl_3$): δ 8.64 (d, J = 8.3 Hz, 1H, H-1), 8.52 (d, J = 2.0 Hz, 1H, H-4), 7.94 (dd, J = 8.3, 2.0 Hz, 1H, H-2), 7.68 (dd, J = 2.9, 1.3 Hz, 1H, thiophene H-2), 7.60 (d, J = 9.1 Hz, 1H, H-11), 7.57 (dd, J = 5.0, 1.3 Hz, 1H, thiophene H-5), 7.43 (dd, J = 5.0, 2.9 Hz, 1H, thiophene H-4), 6.66 (dd, J = 9.1, 2.7 Hz, 1H, H-10), 6.46 (d, J = 2.7 Hz, 1H, H-8), 6.40 (s, 1H, H-6), 3.45 (q, J = 7.1 Hz, 4H, CH_2), 1.26 (t, J = 7.1 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$): δ 183.7 (C-5), 152.3 (C-6a), 150.7 (C-9), 146.7 (C-7a), 141.4 (C-12a), 137.2 (C-3), 132.2 (thiophene C-3), 131.1 (C-11), 130.7 (C-4a), 130.0 (C-12b), 129.1 (C-2), 126.5, 126.3 (thiophene C-4, thiophene C-5), 125.3 (C-11a), 124.6 (C-1), 123.1 (C-4), 121.7 (thiophene C-2), 109.8 (C-10), 105.8 (C-6), 96.4 (C-8), 45.1 (CH_2), 12.6 (CH_3). HRMS (ESI): calculated for $C_{24}H_{21}N_2O_2S$ [M+H$^+$]: 401.1318; found: 401.1309.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: fluorescence · synthetic methods · solvatochromism · Suzuki coupling · two-photon brightness

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