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Imaging Agents

New Red-Emitting Tetrazine-Phenoxazine Fluorogenic Labels for Live-Cell Intracellular Bioorthogonal Labeling Schemes

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Abstract: The synthesis of a set of tetrazine-bearing fluorogenic dyes suitable for intracellular labeling of proteins in live cells is presented. The red excitability and emission properties ensure minimal autofluorescence, while through-bond energy-transfer-based fluorogenicity reduces nonspecific background fluorescence of unreacted dyes. The tetrazine motif efficiently quenches fluorescence of the phenoxazine core, which can be selectively turned on chemically upon bioorthogonal inverse-electron-demand Diels–Alder reaction with proteins modified genetically with strained *trans*-cyclooctenes.

Introduction

Site-specific fluorescent labeling of proteins by means of bioorthogonal tagging schemes has contributed greatly to the better understanding of biomolecular processes.[1] The parallel evolution of bioorthogonal chemistry and genetic encoding techniques led to the emergence of methodologies that enable specific and site-selective fluorescent manipulation of proteins of interest at virtually any position.[2] Fluorescent modulation schemes are often impaired by autofluorescence of naturally occurring fluorophores or nonspecific background fluorescence of unreacted dyes.[3] Very few bioorthogonally applicable fluorescent dyes exist that are membrane permeable and allow intracellular labeling of biomolecules without prior permeabilization.[4] A notable example is presented by the introduction of fluorogenic silicon–rhodamine dyes, with a very attractive mechanism for fluorogenicity.[4b,c] However, data suggests that the success of the concept strongly depends on the hydrophobic–hydrophilic properties of the protein surfaces around the labeling site, and thus, allows less general use.[4b,c] Although autofluorescence can be diminished with dyes that are excitable in the red, near-infrared region of the spectrum, background fluorescence can be efficiently reduced by using fluorogenic dyes (e.g., silicon rhodamines).[1,4b,c,5] Fluorogenic dyes exhibit quenched fluorescence until they react with their specific target. Such chemical reactions transform the quenching moieties; thus fluorescence is reinstated. There are several accounts in the literature presenting fluorogenic dyes, with fewer examples in combination with bioorthogonality.[4b,6] Of the bioorthogonal functions, there are two chemical motifs that efficiently quench fluorescence: azide and tetrazine.[1,5] These two functions are notable because they both act as quenchers and bioorthogonally reactive moieties. Azides are able to diminish fluorescence either by photoinduced electron transfer[6e] or by opening nonradiative relaxation pathways through its fast rotation.[6c,d] Tetrazines, on the other hand, are able to quench fluorescence by energy-transfer processes.[6f–j] There are examples in the literature for tetrazine-based fluorogenic ligations, in which Förster resonance energy transfer (FRET) between various fluorescent frameworks and tetrazine moieties accounts for the quenching process in the context of DNA ligation, lipid staining, or taxol-based microtubule labeling schemes.[6f–h] Apart from FRET, another mechanism, namely, through-bond energy-transfer (TBET), also exists in specifically designed fluorophore–tetrazine constructs.[6i,j] TBET systems require the energy donor and acceptor moieties to be connected through twisted, but otherwise conjugated, linkers.[7] Of the two major energy-transfer mechanisms, TBET is not constrained by spectral overlap between the donor and acceptor moieties.[7] Thus, together with its much more efficient quenching efficiency in comparison with FRET, TBET enables the integration of virtually any kind of fluorescent frameworks as energy donors and tetrazines as acceptors.[6i,j,7]

Phenoxazine-based fluorescent scaffolds, such as resorufin or Nile red congeners, are synthetically easily accessible and have excitation/emission maxima in the red or near-infrared regime. Moreover, phenoxazines can be excited with commercially available laser sources and have brightness and photostability comparable with other dyes in this spectral window.[8] However, due to its high lipophilicity, specific fluorescent labeling with Nile red is often compromised by high background fluorescence. We hypothesize that the latter problem can be efficiently addressed by the development of fluorogenic

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probes and the previously mentioned advantageous characteristics can be fully harvested in the context of cell biology. There are examples in the literature for fluorogenic phenoxazines. In most of these reports, the fluorogenicity is based either on the polarity-sensitive nature of the fluorescent core or on the enzymatic removal of N-acyl substituents.[8e–h] Recently, an azide-quenched benzophenoxazine was also reported.[8i] The most prominent fluorogenic benzophenoxazine derivative is the biarsenic congener. This FlAsH probe is widely utilized in combination with Cys_4-tagged proteins.[8j] To the best of our knowledge, however, neither tetrazine derivatives, nor TBET probes of phenoxazines exist.

Herein, we present the synthesis of two sets of red-emitting phenoxazine–tetrazine TBET dyes suitable for intracellular live-cell imaging. The phenylene and vinylene linkages between the energy-donor phenoxazine and energy-acceptor tetrazine moieties ensure efficient quenching of fluorescence by TBET. Fluorescence of the phenoxazine can be restored chemically upon the bioorthogonally applicable inverse-electron-demand Diels–Alder reaction with proteins modified genetically with strained *trans*-cyclooctenes (TCOs) as the dienophile.

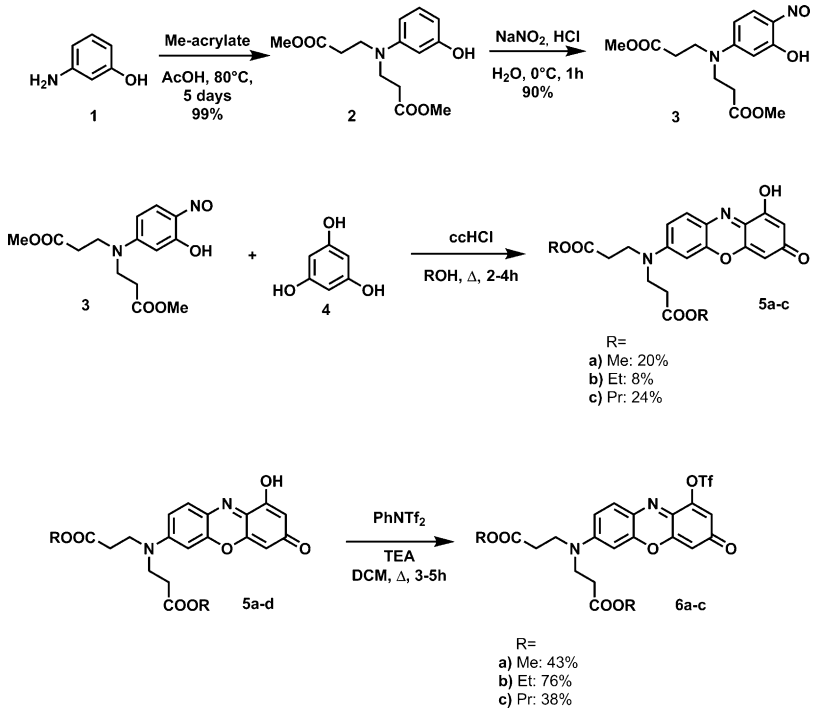
Results and Discussion

To synthesize the phenylene- and vinylene-linked phenoxazine–tetrazine dyes, we have devised synthetic routes in which the phenyl- and vinyltetrazine frames are installed onto the phenoxazine core by Suzuki- or Heck-type cross-coupling reactions, respectively. In accordance with our preliminary results, it is reported in the literature that phenoxazine-type of dyes are prone to form nonfluorescent dimers and/or H-type aggregates in aqueous media through π–π stacking interactions.[9] To decrease this tendency, we designed and synthesized N,N-disubstituted derivatives with different steric bulks. In the first step, 3-hydroxyaniline was reacted with methyl acrylate to give the corresponding dipropionate, **2**. Compound **2** was subjected to nitrosation to form **3** in excellent yield. Nitroso compound **3** was allowed to react with phloroglucinol (**4**) by using different alcohols as reaction media to form compounds **5a**–**c**. Triflation of **5a**–**c** was effected by treatment with *N*-phenyl bis(trifluoromethanesulfonimide) to give compounds **6a**–**c** (Scheme 1).

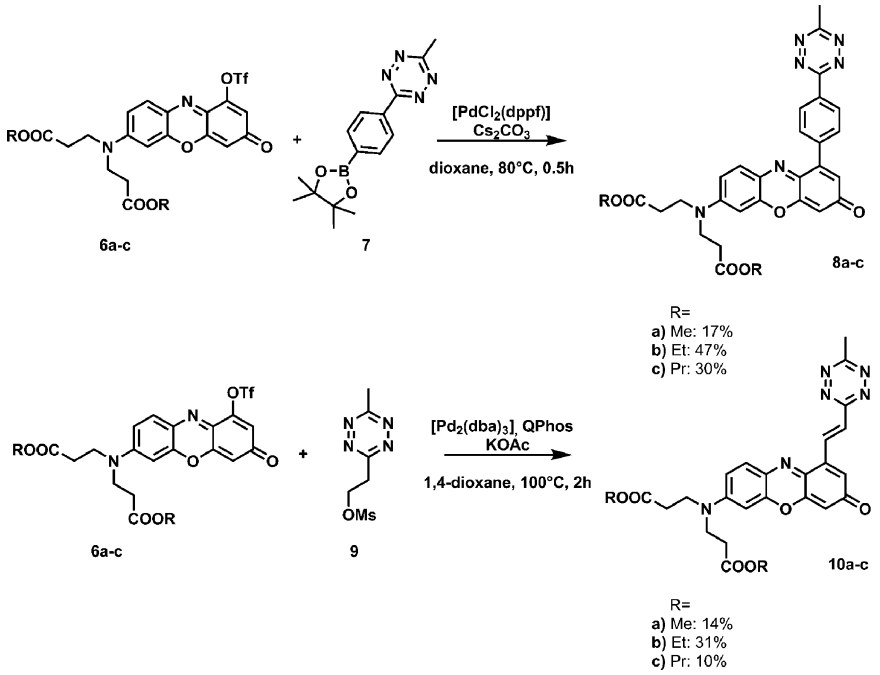
Parallel to this, methyltetrazine boronic acid pinacol ester (**7**) was synthesized from 3-(4-bromophenyl)-6-methyl-1,2,4,5-tetrazine obtained by a modified procedure reported by Chenoweth et al.[10] and bis(pinacolato)diboron in 40% yield. Suzuki cross-coupling reactions of **6a**–**c** and **7** resulted in phenylene-linked TBET dyes **8a**–**c** in moderate yields (Scheme 2).

For the vinylene-linked TBET dye series, vinyltetrazine precursor (**9**), was obtained from 2-(6-methyl-1,2,4,5-tetrazin-3-yl)ethan-1-ol, upon mesylation with methanesulfonyl chloride (MsCl).[11] Palladium-catalyzed Heck coupling of **6a**–**c** and **9** afforded vinylene-linked TBET dyes **10a**–**c** in acceptable yields (Scheme 2).

Next, we characterized the spectroscopic properties of the new phenoxazine–tetrazines. As expected, the absorption and emission maxima of the two dye series were found to be at $\lambda = 590$ and 600 nm and $\lambda = 625$ and 635 nm for the phenylene and vinylene series, respectively. The absorption spectra showed a shoulder in each case, which indicated transitions that were characteristic of the phenoxazine and tetrazine moieties (see the Supporting Information). To assess the fluorogen-



Scheme 1. Synthesis of phenoxazine triflates, **6a**–**c**. TEA = triethylamine.



Scheme 2. Synthesis of phenoxazine–tetrazines **8** and **10**. Tf = trifluoromethanesulfonyl, dppf = 1,1′-bis(diphenylphosphino)ferrocene, dba = dibenzylideneacetone, QPhos = 1,2,3,4,5-pentaphenyl-1′-(di-*tert*-butylphosphino)ferrocene.

ic performances, we used a hydrophilic TCO derivative, **OxTCO**, as a dienophile partner that triggered fluorescence upon reacting with the tetrazine.[12] To our delight, all compounds showed appreciable increase in fluorescence emission intensity upon reaction with the TCO; however, their fluorogenic performance varied considerably (from 8- to 275-fold). In general, the phenylene series **8** had better fluorogenic performances than vinylene-linked dyes **10**. On the other hand, series **10** reacted about 10 times faster with **OxTCO** than series **8** (Figure 1). Within the series, the fluorogenic performances also showed great variabilities, that is, greater steric demand of the N substituents resulted in a larger fluorescence enhancement (FE) being observed (Figure 1, Table 1, and the Supporting Information). This can be explained on the basis of aggregation tendencies. Bulkier substituents prevent the formation of nonfluorescent dimers; thus more intense final fluorescence signals are achieved. To determine ETEs, we selected the two best-performing tetrazines, since dimer formation in the case of **8a**,**b** and **10a**,**b** compromised the fluorescence performances. Thus, we prepared **OxTCO** conjugates of **8c** and **10c**. In both cases, the primary reaction products readily oxidized to the corresponding benzopyridazines, giving rise to diastereomeric mixtures of **8c-OxTCO** and **10c-OxTCO**. We determined relative quantum yields for **8c**, **10c**, and the **OxTCO** adducts and concluded that the ETEs were 99 and 93%, which further supported that the energy transfer took place through the TBET mechanism (Table 1). It should also be noted that the phenylene linker provides a more advantageous platform for the energy-transfer process.

Table 1. FE values, quantum yields (Φ_F), and energy-transfer efficiencies (ETEs) of fluorogenic dyes **8** and **10**.[a]

Dye	FE	Φ_F [%][b]	ETE [%][c]
8a	8	n.a.	n.a.
8b	70	n.a.	n.a.
8c	275	0.15	99
10a	13	n.a.	n.a.
10b	35	n.a.	n.a.
10c	53	0.25	93
8c-OxTCO	n.a.	10.6	n.a.
10c-OxTCO	n.a.	3.75	n.a.

[a] In phosphate-buffered saline (PBS). [b] Relative to rhodamine 6G (0.95). [c] ETE = $1-\Phi_F$(tetrazine)/Φ_F(conjugate).

Next, we were curious about the protein-labeling abilities of the dyes in live-cell applications. To this end, we modified green fluorescent protein (GFP) at position 39 with TCO* GFP(Y39$^{TAG\rightarrow TCO*}$) by genetic code expansion.[13] GFP(Y39$^{TAG\rightarrow TCO*}$) was expressed in mammalian HEK293T cells and live-cell labeling was performed with dyes **8a–c** and **10a–c** for 1 h, then cells were washed and subjected to confocal microscopy imaging (Figure 2). In all cases, the dyes showed efficient specific intracellular labeling of GFP, which suggested that the phenoxazine–tetrazine dyes were cell-membrane permeable. By contrast, cells that did not express GFP had no appreciable labeling; this indicated that unreacted dyes were efficiently quenched and did not contribute to nonspecific background fluorescence when only physically adsorbed on hydrophobic surfaces. This is in contrast to commonly used dyes such as rhodamines or Nile dyes (Figure 2).

Dyes containing substituents with smaller steric demands could label the intracellular proteins more efficiently. If we consider that intracellular hydrolases convert the individual members of the two series into identical species, it seems reasona-

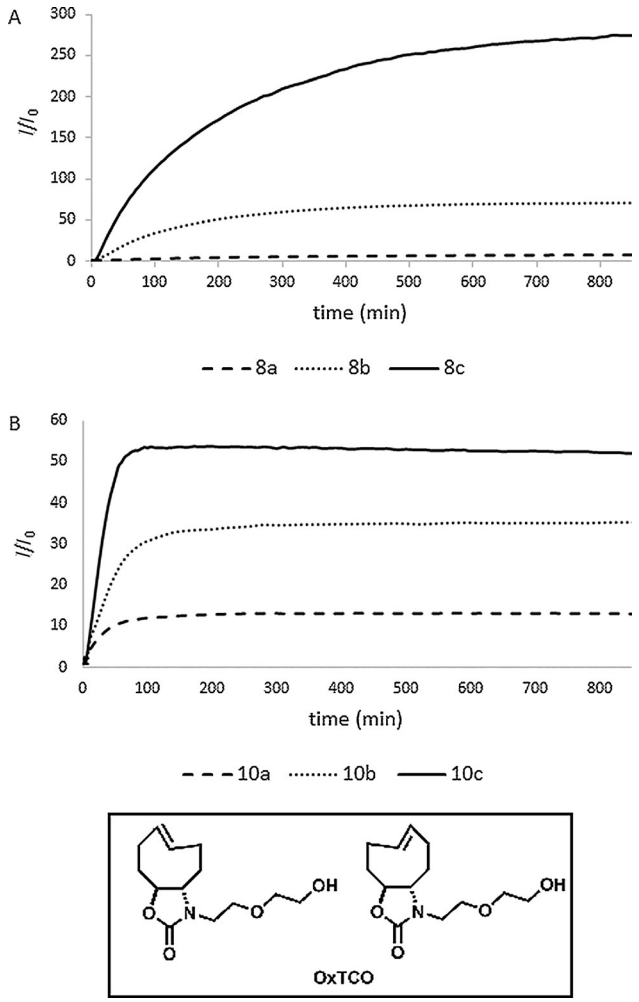


Figure 1. Time-dependent fluorescence changes for series **8** and **10** in the presence of excess **OxTCO** (excited and detected at the wavelengths of maximal absorption and emission).

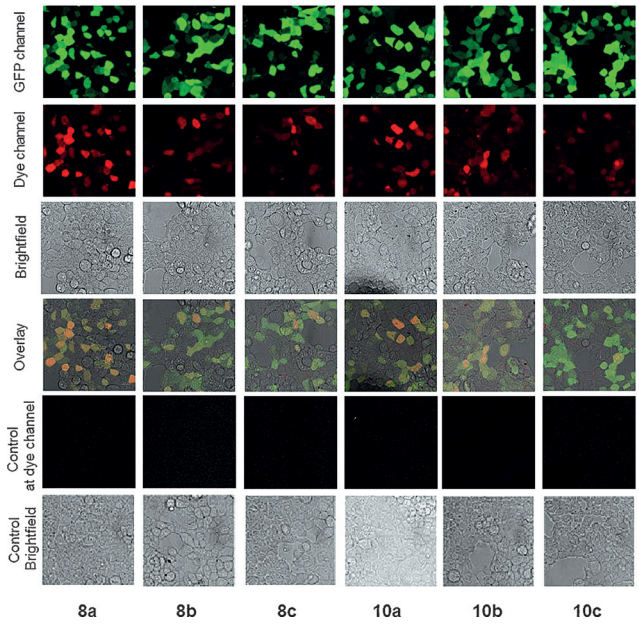


Figure 2. Confocal microscopy images of GFP(Y39$^{TAG\rightarrow TCO*}$) expressed in HEK293T cells treated live with 5 μM **8a–c** and **10a–c** for 1 h. Controls show confocal microscopy images of nontransfected HEK293T cells treated live with 5 μM **8a–c** and **10a–c** for 1 h.

ble to state that different membrane permeabilities can account for this observation. Dyes with smaller steric demands can traffic through the membrane more efficiently.

Conclusion

We synthesized two series of phenoxazine-linked tetrazine fluorogenic scaffolds. Phenylene or vinylene linkages between the phenoxazine and tetrazine modules ensured efficient quenching of the phenoxazine fluorescence by the tetrazine motif through the TBET mechanism, which could be restored upon a bioorthogonal inverse-electron-demand Diels–Alder reaction with TCOs. Of the two sets, the phenylene-linked TBET systems showed excellent fluorogenicity upon reaction, especially when their tendency to form nonfluorescent aggregates was prevented by sterically demanding substituents. The red excitation and emission properties make these dyes ideal candidates for live-cell labeling studies because low autofluorescence is expected in this spectral window. Moreover, the TBET-based fluorogenicity efficiently reduces nonspecific background fluorescence of unreacted dyes. We also tested the applicability of the dyes in live-cell labeling experiments by using intracellular GFP(Y39$^{TAG\rightarrow TCO*}$)-expressing mammalian cells. It could be said that both sets of dyes, especially those with smaller steric demand, were membrane-permeable and suitable for specific labeling of target proteins with very low background fluorescence. In this respect, it seems irrelevant whether the dyes form nonfluorescent aggregates because their overall performance is mainly dependent on their membrane permeability, as governed by the steric demand of the substituents.

Such bioorthogonally applicable fluorogenic dyes that are excited and emit light in the red regime are extremely useful in fluorescent imaging techniques. The specific reactivity can be further exploited in the field of super-resolution fluorescent microscopy; an application currently under investigation in our laboratory.

Experimental Section

General

Unless otherwise indicated, all starting materials were obtained from commercial suppliers (Sigma–Aldrich, Fluka, Merck, Alfa Aesar, Reanal, Molar Chemicals, Fluorochem) and used without further purification. Analytical TLC was performed on silica gel 60 F254 precoated aluminum TLC plates from Merck. Preparative TLC was performed on Kieselgel 60 F254 preparative TLC plates. Semipreparative HPLC was performed on a Hanbon Sci. & Tech. NP 7010C instrument with a Gemini® 5 μm C18 110 Å, 150×21.20 mm LC column; detection was performed with an ECOM Flash 14 DAD 600 UV/Vis detector. The flow rate was 15 mL min^{-1} with a linear gradient elution (0 min 5% B; 3 min 5% B; 6 min 50% B; 15 min

70% B; 20 min 95% B; 25 min 95% B) with eluents A (100% water) and B (20% water, 80% acetonitrile), with 0.1% trifluoracetic acid in the case of compound **8a**. Flash column chromatography was performed on Teledyne Isco CombiFlash® R_f^+ automated flash chromatography apparatus by using silica gel (25–40 μm) from Zeochem. Fluorescence measurements were performed on a Jasco FP 8300 spectrofluorometer. Microwave experiments were carried out on a Monowave 300 microwave reactor. NMR spectra were recorded on Varian Inova 500 MHz and Varian 600 MHz NMR spectrometers. Chemical shifts (δ) are given in parts per million (ppm) by using solvent signals as the reference.[14] Coupling constants (J) are reported in Hertz (Hz). Splitting patterns are designated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of a doublet), br s (broad singlet). Analytical RP-HPLC-UV/Vis-MS experiments were performed on a SHIMADZU LCMS-2020 system by using a Gemini C18 column (100×2.00 mm I.D.) with 5 μm silica (110 pore size) as a stationary phase with a photodiode array UV/Vis (λ = 220–800 nm) and an ESI-MS detector. Linear gradient elution (0 min 0% B; 1.0 min 100% B; 3.5 min 100% B; 4.5 min 0% B; 5.0 min 0% B) with eluents A (2% NH_4HCO_3, 5% acetonitrile, and 93% water) and B (2% NH_4HCO_3, 80% acetonitrile, and 18% water) was used at a flow rate of 0.5 mL min^{-1} at 30 °C. The samples were dissolved in acetonitrile. Time-dependent fluorescence measurements were conducted in PBS with 1% DMSO (1.0 μM dye with 100 equiv of **OxTCO**)

Dimethyl 3,3′-[(3-hydroxyphenyl)azanediyl]dipropionate (2)[8h]

Commercially available 3-aminophenol (**1**; 5.00 g, 45.9 mmol, 1.0 equiv) was heated in methyl acrylate (15.8 g, 16.5 mL, 183.5 mmol, 4.0 equiv) and acetic acid (1.38 g, 1.31 mL, 22.9 mmol, 0.5 equiv) at 80 °C for 5 days. After 5 days, the volatile components were removed under reduced pressure and **2** was obtained as a brown oil (12.9 g, 99%). The product was used without further purification. ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 6.94 (t, J = 7.9 Hz, 1H), 6.12 (d, J = 9.1 Hz, 1H), 6.09 (s, 1H), 6.08 (d, J = 7.3 Hz, 1H), 3.60 (s, 6H), 3.51 (t, J = 7.0 Hz, 4H), 2.53 ppm (t, J = 7.0 Hz, 4H); ^{13}C NMR (126 MHz, [D$_6$]DMSO): δ = 172.5, 158.9, 148.5, 130.3, 104.3, 103.9, 99.8, 51.8, 46.7, 32.2 ppm; HRMS (ESI): m/z calcd for $C_{14}H_{20}NO_5^+$ $[M+H]^+$: 282.1336; found: 282.1341.

Dimethyl 3,3′-[(3-hydroxy-4-nitrosophenyl)azanediyl]dipropionate (3)[15]

Compound **2** (12.8 g, 45.5 mmol 1.0 equiv) was dissolved in water (200 mL) and 37% HCl (10 mL), and cooled to 0 °C. A solution of $NaNO_2$ (3.14 g, 45.5 mmol, 1.0 equiv) in water (50 mL) was added dropwise over a period of 10 min. The solution was stirred for an additional 2 h, during which time it was allowed to warm to room temperature. The solution was extracted with EtOAc, then the organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo to give **3** as a brown solid (12.7 g, 90%), which was used without further purification. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.51 (d, J = 9.8 Hz, 1H), 6.56 (d, J = 9.7 Hz, 1H), 5.70 (s, 1H), 3.82 (t, J = 6.9 Hz, 4H), 3.70 (s, 6H), 2.68 ppm (t, J = 7.0 Hz, 4H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 171.0, 167.5, 156.9, 150.0, 136.2, 112.5, 97.1, 52.1, 47.3, 32.4 ppm; HRMS (ESI): m/z calcd for $C_{14}H_{19}N_2O_6^+$ $[M+H]^+$: 311.1238; found: 311.1243.

Dimethyl 3,3′-[(1-hydroxy-3-oxo-3*H*-phenoxazin-7-yl)azanediyl]dipropionate (5a)

Compound **3** (4.00 g, 12.90 mmol, 1.0 equiv) and phloroglucinol dihydrate (2.09 g, 12.90 mmol, 1.0 equiv) was dissolved in methanol (150 mL) and 37% HCl (2 mL) was added. The solution was heated at reflux for 4 h. After 4 h, the solution was cooled to room temperature and concentrated in vacuo. The crude product was dissolved in CH_2Cl_2 (400 mL) and washed with brine (5×200 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo to give **5a** as a purple solid (0.970 g, 20%). The product was used without further purification. R_f (CH_2Cl_2/MeOH 9:1 v/v): 0.7; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.52 (d, J = 9.0 Hz, 1H), 6.66 (d, J = 8.8 Hz, 1H), 6.46 (s, 1H), 6.14 (s, 1H), 6.03 (s, 1H), 3.79 (t, J = 6.9 Hz, 4H), 3.71 (t, J = 15.3 Hz, 6H), 2.67 ppm (t, J = 6.9 Hz, 4H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 186.4, 171.5, 154.9, 151.0, 148.5, 147.8, 137.5, 131.3, 124.2, 110.1, 104.4, 97.4, 52.0, 47.0, 32.1 ppm; HRMS (ESI): m/z calcd for $C_{20}H_{21}N_2O_7^+$ $[M+H]^+$: 401.1343; found: 401.1349.

Diethyl 3,3′-[(1-hydroxy-3-oxo-3*H*-phenoxazin-7-yl)azanediyl]dipropionate (5b)[16]

Compound **3** (4.00 g, 12.90 mmol, 1.0 equiv) and phloroglucinol dihydrate (2.09 g, 12.90 mmol, 1.0 equiv) were dissolved in ethanol (150 mL) and 37% HCl (2 mL) was added. The solution was heated at reflux for 2 h. After 2 h, the solution was cooled to room temperature and concentrated in vacuo. The crude product was dissolved in CH_2Cl_2 (400 mL) and washed with brine (5×200 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo to give **5b** as a purple product (0.455 g, 8%). The product was used without further purification. R_f (CH_2Cl_2/MeOH 9:1 v/v): 0.7; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.68 (d, J = 9.1 Hz, 1H), 6.80 (d, J = 8.7 Hz, 1H), 6.69 (s, 1H), 6.54 (s, 1H), 6.31 (s, 1H), 4.17 (q, J = 6.9 Hz, 4H), 3.84 (t, J = 6.6 Hz, 4H), 2.67 (t, J = 6.6 Hz, 4H), 1.27 ppm (t, J = 7.0 Hz, 6H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 183.0, 170.9, 152.7, 149.8, 149.4, 147.5, 134.7, 133.2, 126.8, 121.9, 111.7, 105.6, 96.9, 61.2, 47.2, 32.3, 14.1 ppm; HRMS (ESI): m/z calcd for $C_{22}H_{25}N_2O_7^+$ $[M+H]^+$: 429.1656; found: 429.1662.

Dipropyl 3,3′-[(1-hydroxy-3-oxo-3*H*-phenoxazin-7-yl)azanediyl]dipropionate (5c)

Compound **3** (4.00 g, 12.90 mmol, 1.0 equiv) and phloroglucinol dihydrate (2.09 g, 12.90 mmol, 1.0 equiv) were dissolved in 1-propanol (150 mL) and 37% HCl (2 mL). The solution was heated at reflux for 4 h. After 4 h, the solution was cooled to room temperature and concentrated under reduced pressure. The crude product was dissolved in CH_2Cl_2 (400 mL) and washed with brine (5× 200 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo to give **5c** as a purple solid (1.35 g, 24%), which was used subsequently without further purification. R_f (CH_2Cl_2/MeOH 9:1 v/v): 0.7; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.51 (d, J = 9.0 Hz, 1H), 6.66 (d, J = 8.3 Hz, 1H), 6.46 (s, 1H), 6.13 (s, 1H), 6.02 (s, 1H), 4.06 (t, J = 6.6 Hz, 4H), 3.78 (t, J = 6.8 Hz, 4H), 2.66 (t, J = 6.9 Hz, 4H), 1.65 (q, J = 7.1 Hz, 4H), 0.93 ppm (t, J = 7.4 Hz, 6H); ^{13}C NMR (126 MHz, CDCl3): δ = 186.5, 171.2, 155.2, 151.1, 148.5, 147.7, 137.3, 131.4, 124.3, 110.2, 104.2, 97.4, 66.7, 47.0, 32.3, 21.9, 10.3 ppm; HRMS (ESI): m/z calcd for $C_{24}H_{29}N_2O_7^+$ $[M+H]^+$: 457.1969; found: 457.1975.

Dimethyl 3,3′-[(3-oxo-1-{[(trifluoromethyl)sulfonyl]oxy}-3*H*-phenoxazin-7-yl)azanediyl]dipropionate (6a)

A mixture of compound **5a** (950 mg, 2.375 mmol 1.0 equiv) and commercially available *N*-phenyl bis(trifluoromethanesulfonimide) (848 mg, 2.375 mmol, 1.0 equiv) was dissolved in CH_2Cl_2 (100 mL). TEA (2.40 g, 3.31 mL, 23.75 mmol, 10.0 equiv) was added and the solution was heated at reflux for 5 h. After 5 h, the volatile compounds were removed under reduced pressure and product was purified by flash column chromatography on silica gel (0–50 v/v% EtOAc in hexane in 20 min, then 50 v/v% EtOAc in hexane for 20 min) to give **6a** as a green solid (550 mg, 43%). R_f (CH_2Cl_2/MeOH 20:1 v/v): 0.6; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.68 (d, J = 9.2 Hz, 1H), 6.77 (dd, J = 9.2, 2.6 Hz, 1H), 6.67 (d, J = 1.8 Hz, 1H), 6.52 (d, J = 2.6 Hz, 1H), 6.28 (d, J = 1.8 Hz, 1H), 3.84 (t, J = 7.0 Hz, 4H), 3.72 (s, 6H), 2.69 ppm (t, J = 7.0 Hz, 4H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 182.9, 171.3, 152.4, 149.7, 149.4, 147.4, 135.0, 133.2, 126.6, 122.1, 111.5, 105.7, 96.9, 52.1, 47.2, 32.0 ppm; IR (neat): $\tilde{\nu}_{max}$ = 1734, 1605, 1491, 1368, 1207, 1142 cm^{-1}; HRMS (ESI): *m/z* calcd for $C_{21}H_{20}F_3N_2O_9S^+$ $[M+H]^+$: 533.0836; found: 533.0842.

Diethyl 3,3′-[(3-oxo-1-{[(trifluoromethyl)sulfonyl]oxy}-3*H*-phenoxazin-7-yl)azanediyl]dipropionate (6b)

A mixture of **5b** (455 mg, 1.063 mmol, 1.0 equiv) and commercially available *N*-phenyl bis(trifluoromethanesulfonimide) (569 mg, 1.595 mmol, 1.5 equiv) was dissolved in CH_2Cl_2 (50 mL). TEA (1.074 g, 1.48 mL, 10.63 mmol 10.0 equiv) was added and the solution was heated at reflux for 3 h. After 3 h, CH_2Cl_2 and TEA were removed under reduced pressure and the product was purified by flash chromatography on silica gel (0–50 v/v% EtOAc in hexane in 20 min, then 50 v/v% EtOAc in hexane for 20 min) to afford **6b** as a green solid (450 mg, 76%). R_f (CH_2Cl_2/MeOH 20:1 v/v): 0.6; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.68 (d, J = 9.1 Hz, 1H), 6.80 (d, J = 8.1 Hz, 1H), 6.69 (s, 1H), 6.54 (s, 1H), 6.31 (s, 1H), 4.17 (q, J = 6.9 Hz, 4H), 3.84 (t, J = 6.6 Hz, 4H), 2.67 (t, J = 6.6 Hz, 4H), 1.27 ppm (t, J = 7.0 Hz, 6H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 183.0, 170.9, 152.7, 149.8, 149.4, 147.5, 134.7, 133.2, 126.8, 123.1, 121.9, 111.7, 105.6, 96.9, 61.2, 47.2, 32.3, 14.1 ppm; IR (neat): $\tilde{\nu}_{max}$ = 1730, 1605, 1491, 1373, 1211, 1142 cm^{-1}; HRMS (ESI): *m/z* calcd for $C_{23}H_{24}F_3N_2O_9S^+$ $[M+H]^+$: 561.1149; found: 561.1155.

Dipropyl 3,3′-[(3-oxo-1-{[(trifluoromethyl)sulfonyl]oxy}-3*H*-phenoxazin-7-yl)azanediyl]dipropionate (6c)

A mixture of **5c** (200 mg, 0.439 mmol, 1.0 equiv) and commercially available N-phenyl bis(trifluoromethanesulfonimide) (157 mg, 0.439 mmol, 1.0 equiv) was dissolved in CH_2Cl_2 (20 mL). TEA (443 mg, 0.61 mL, 4.39 mmol, 10.0 equiv) was added and the solution was heated at reflux for 4.5 h. After 4.5 h, the volatile compounds were removed under reduced pressure and the product was purified by flash chromatography on silica gel (0–50 v/v% EtOAc in hexane in 20 min, then 50 v/v% EtOAc in hexane for 20 min) to give **6c** as a green solid (97 mg, 38%). R_f (CH_2Cl_2/MeOH 20:1 v/v): 0.6; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.69 (d, J = 9.2 Hz, 1H), 6.79 (d, J = 9.1 Hz, 1H), 6.69 (s, 1H), 6.54 (s, 1H), 6.30 (s, 1H), 4.07 (t, J = 6.7 Hz, 4H), 3.85 (t, J = 6.8 Hz, 4H), 2.68 (t, J = 6.8 Hz, 4H), 1.66 (q, J = 7.0 Hz, 4H), 0.94 ppm (t, J = 7.4 Hz, 6H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 182.9, 171.0, 152.5, 149.7, 149.5, 147.4, 134.9, 133.2, 126.7, 122.0, 111.6, 105.7, 96.9, 66.8, 47.2, 32.3, 21.9, 10.3; IR (neat): $\tilde{\nu}_{max}$ = 2924, 1732, 1603, 1452, 1364, 1213, 1144 cm^{-1}; HRMS (ESI): *m/z* calcd for $C_{25}H_{28}F_3N_2O_9S^+$ $[M+H]^+$: 589.1462; found: 589.1468.

3-Methyl-6-[4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-phenyl]-1,2,4,5-tetrazine (7)

A mixture of 3-(4-bromophenyl)-6-methyl-1,2,4,5-tetrazine[10] (105 mg, 0.414 mmol, 1.0 equiv), commercially available bis(pinacolato)diboron (117 mg, 0.456 mmol, 1.1 equiv), [$PdCl_2$(dppf)] (15 mg, 0.021 mmol, 0.05 equiv), and KOAc (61 mg, 0.622 mmol, 1.5 equiv) were suspended in 1,4-dioxane (5 mL), and the suspension was heated at reflux for 2 h. After 2 h, the solution was cooled to room temperature, filtered through a Celite pad, and concentrated in vacuo. The residue was dissolved in CH_2Cl_2 (20 mL) and washed with water (3×50 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography on silica gel (0–30 v/v EtOAc in hexane for 6 min, then 30 v/v% EtOAc in hexane for 15 min) to give **7** as a red solid (32 mg, 40%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.54 (d, J = 7.8 Hz, 2H), 7.98 (d, J = 7.8 Hz, 2H), 3.06 (s, 3H), 1.35 ppm (s, 12H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 167.2, 164.1, 135.4, 134.0, 126.9, 84.1, 24.8, 21.1 ppm; ^{11}B NMR (160 MHz, $CDCl_3$): δ = 30.0 ppm; HRMS (ESI): *m/z* calcd for $C_{15}H_{20}BN_4O_2^+$ $[M+H]^+$: 299.1674; found: 299.1679.

Dimethyl 3,3′-({1-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]-3-oxo-3*H*-phenoxazin-7-yl}azanediyl)dipropionate (8a)

Compound **6a** (33 mg, 0.062 mmol, 1.0 equiv), **7** (18 mg, 0.062 mmol, 1.0 equiv), [$PdCl_2$(dppf)] (2.3 mg, 0.003 mmol, 0.05 equiv), and Cs_2CO_3 (61 mg, 0.186 mmol, 3.0 equiv) were suspended in 1,4-dioxane (7.5 mL). The suspension was stirred for 30 min at 80 °C, after which time it was cooled to room temperature and CH_2Cl_2 (30 mL) was added. The organic layer was washed with brine (3×40 mL), separated, concentrated in vacuo, and purified by semipreparative HPLC (linear gradient elution). For fluorescence measurements, the product was further purified by preparative TLC (CH_2Cl_2/methanol 20:1 v/v) to give **8a** as a red solid (6.0 mg, 17%). R_f (CH_2Cl_2/MeOH 20:1 v/v%): 0.5; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.69 (d, J = 7.9 Hz, 1H), 7.81 (d, J = 7.9 Hz, 1H), 7.57 (d, J = 9.0 Hz, 1H), 6.92 (s, 1H), 6.70 (d, J = 7.2 Hz, 1H), 6.53 (s, 1H), 6.40 (s, 1H), 3.82 (t, J = 6.6 Hz, 4H), 3.72 (s, 6H), 3.13 (s, 3H), 2.69 ppm (t, J = 6.4 Hz, 4H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 184.9, 171.5, 167.3, 164.0, 151.3, 150.4, 146.6, 144.2, 141.0, 139.9, 132.5, 131.9, 131.8, 131.0, 127.4, 126.6, 110.6, 106.3, 96.8, 52.0, 47.1, 32.1, 21.2 ppm; IR (neat) $\tilde{\nu}_{max}$ = 2922, 1728, 1609, 1587, 1402, 1364, 1248, 1130, 851 cm^{-1}; HRMS (ESI): *m/z* calcd for $C_{29}H_{27}N_6O_6^+$ $[M+H]^+$: 555.1987; found: 555.1992.

Diethyl 3,3′-({1-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]-3-oxo-3*H*-phenoxazin-7-yl}azanediyl)dipropionate (8b)

Compound **6b** (20 mg, 0.036 mmol, 1.0 equiv), **7** (11 mg, 0.036 mmol, 1.0 equiv), [$PdCl_2$(dppf)] (1.3 mg, 0.002 mmol, 0.05 equiv), and Cs_2CO_3 (35 mg, 0.107 mmol, 3.0 equiv) were suspended in 1,4-dioxane (5 mL). The suspension was stirred for 30 min at 80 °C, after which time it was cooled to room temperature, and CH_2Cl_2 (30 mL) was added. The organic layer was washed with brine (3×40 mL), separated, concentrated in vacuo, and purified by semipreparative HPLC (linear gradient elution) to provide **8b** as a red solid (9.8 mg, 47%). R_f (CH_2Cl_2/MeOH 20:1 v/v): 0.5; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.68 (d, J = 8.0 Hz, 2H), 7.81 (d, J = 8.0 Hz, 2H), 7.56 (d, J = 9.1 Hz, 1H), 6.92 (s, 1H), 6.71 (d, J = 7.2 Hz, 1H), 6.54 (s, 1H), 6.39 (s, 1H), 4.17 (q, J = 6.9 Hz, 4H), 3.82 (t, J = 6.5 Hz, 4H), 3.12 (s, 3H), 2.67 (t, J = 6.5 Hz, 4H), 1.27 ppm (t, J = 7.1 Hz, 6H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 184.9, 171.1, 167.3, 163.9, 151.4, 150.4, 146.6, 144.1, 140.9, 139.9, 132.5, 131.8, 131.8,

131.0, 127.5, 126.6, 110.6, 106.3, 96.8, 61.0, 47.0, 32.4, 21.2, 14.1 ppm; IR (neat) $\tilde{\nu}_{max}$ = 1722, 1609, 1585, 1398, 1362, 1246, 1194, 1130, 851, 802 cm^{-1}; HRMS (ESI): *m/z* calcd for $C_{31}H_{31}N_6O_6^+$ $[M+H]^+$: 583.2300; found: 583.2305.

Dipropyl 3,3′-({1-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]-3-oxo-3*H*-phenoxazin-7-yl}azanediyl)dipropionate (8c)

Compound **6c** (20 mg, 0.034 mmol, 1.0 equiv), **7** (10 mg, 0.034 mmol, 1.0 equiv), [$PdCl_2$(dppf)] (1.2 mg, 0.002 mmol, 0.05 equiv), and Cs_2CO_3 (33 mg, 0.102 mmol, 3.0 equiv) were suspended in 1,4-dioxane (10 mL). The suspension was stirred for 30 min at 80 °C, after which time it was cooled to room temperature and CH_2Cl_2 (30 mL) was added. The organic layer was washed with brine (3×40 mL), separated, concentrated in vacuo, and purified by semipreparative HPLC (linear gradient elution) to give **8c** as a red solid (6.3 mg, 30%). R_f (CH_2Cl_2/MeOH 20:1 v/v): 0.5; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.67 (d, *J* = 8.2 Hz, 2H), 7.80 (d, *J* = 8.2 Hz, 2H), 7.56 (d, *J* = 9.1 Hz, 1H), 6.91 (d, *J* = 1.9 Hz, 1H), 6.71 (dd, *J* = 9.1, 2.3 Hz, 1H), 6.54 (d, *J* = 2.2 Hz, 1H), 6.39 (d, *J* = 1.8 Hz, 1H), 4.07 (t, *J* = 6.7 Hz, 4H), 3.82 (t, *J* = 6.9 Hz, 4H), 3.12 (s, 3H), 2.68 (t, *J* = 6.9 Hz, 4H), 1.71–1.62 (m, 4H), 0.94 ppm (t, *J* = 7.2 Hz, 6H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 184.9, 171.2, 167.3, 163.9, 151.4, 150.5, 146.6, 144.1, 140.8, 139.9, 132.5, 131.8, 131.7, 131.0, 127.5, 126.6, 110.7, 106.3, 96.8, 66.7, 47.1, 32.3, 21.9, 21.2, 10.3 ppm; IR (neat) $\tilde{\nu}_{max}$ = 1726, 1603, 1402, 1362, 1263, 1186, 1128 cm^{-1}; HRMS (ESI): *m/z* calcd for $C_{33}H_{35}N_6O_6^+$ $[M+H]^+$: 611.2613; found: 611.2618.

Dimethyl 3,3′-({1-[2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl]-3-oxo-3*H*-phenoxazin-7-yl}azanediyl)(*E*)-dipropionate (10a)

Compound **6a** (45 mg, 0.085 mmol, 1.0 equiv), **9** (129 mg, 0.592 mmol, 7.0 equiv), [Pd_2(dba)$_3$] (7.7 mg, 0.008 mmol, 0.1 equiv), QPhos (6.0 mg, 0.008 mmol, 0.1 equiv), and KOAc (66 mg, 0.592 mmol, 8.0 equiv) were suspended in 1,4-dioxane (2 mL). The suspension was stirred for 120 min at 100 °C in a sealed vial, after which time it was cooled to room temperature, the volatile components were removed in vacuo, and CH_2Cl_2 (30 mL) was added. The organic layer was then washed with water (3×40 mL), separated, concentrated in vacuo, and purified by semipreparative HPLC (linear gradient elution). For fluorescence measurements, the product was further purified by preparative TLC (CH_2Cl_2/methanol 20:1 v/v) to give **10a** as a black solid (2.1 mg, 14%). R_f (CH_2Cl_2/MeOH 20:1 v/v): 0.5; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.90 (d, *J* = 16.4 Hz, 1H), 7.76 (d, *J* = 16.5 Hz, 1H), 7.72 (d, *J* = 9.1 Hz, 1H), 7.18 (s, 1H), 6.76 (d, *J* = 6.9 Hz, 1H), 6.52 (s, 1H), 6.37 (s, 1H), 3.83 (t, *J* = 6.9 Hz, 4H), 3.73 (s, 6H), 3.09 (s, 3H), 2.70 ppm (t, *J* = 6.9 Hz, 4H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 185.0, 171.5, 166.6, 164.4, 151.3, 150.5, 146.8, 140.8, 138.3, 134.2, 132.5, 129.1, 126.4, 125.9, 110.8, 107.0, 96.9, 52.0, 47.1, 32.1, 21.2 ppm; IR (neat) $\tilde{\nu}_{max}$ = 2922, 1726, 1601, 1578, 1358, 1256, 1165 cm^{-1}; HRMS (ESI): *m/z* calcd for $C_{25}H_{25}N_6O_6^+$ $[M+H]^+$: 505.1831; found: 505.1836.

Diethyl 3,3′-({1-[2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl]-3-oxo-3*H*-phenoxazin-7-yl}azanediyl)(*E*)-dipropionate (10b)

Compound **6b** (30 mg, 0.054 mmol, 1.0 equiv), **9** (58 mg, 0.268 mmol, 5.0 equiv), [Pd_2(dba)$_3$] (4.9 mg, 0.005 mmol, 0.1 equiv), QPhos (3.8 mg, 0.005 mmol, 0.1 equiv), and KOAc (32 mg, 0.321 mmol, 6.0 equiv) were suspended in 1,4-dioxane (2 mL). The suspension was stirred for 120 min at 100 °C in a sealed vial, after which time it was cooled to room temperature, the volatile components were removed in vacuo, and CH_2Cl_2 (30 mL) was added. The organic layer was washed with water (3×40 mL), separated, concentrated in vacuo, and purified by semipreparative HPLC (linear gradient elution). For fluorescence measurements, the product was further purified by preparative TLC (CH_2Cl_2/methanol 20:1 v/v) to give **10b** as a black solid (8.8 mg, 31%). R_f (CH_2Cl_2/MeOH 20:1 v/v): 0.5; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.90 (d, *J* = 16.4 Hz, 1H), 7.76 (d, *J* = 16.5 Hz, 1H), 7.71 (d, *J* = 8.9 Hz, 1H), 7.17 (s, 1H), 6.77 (d, *J* = 7.1 Hz, 1H), 6.53 (s, 1H), 6.37 (s, 1H), 4.18 (q, *J* = 7.0 Hz, 4H), 3.83 (t, *J* = 6.7 Hz, 4H), 3.09 (s, 3H), 2.68 (t, *J* = 6.8 Hz, 4H), 1.28 ppm (t, *J* = 7.1 Hz, 6H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 185.0, 171.1, 166.6, 164.4, 151.4, 150.5, 146.9, 140.7, 138.3, 134.2, 132.5, 129.1, 126.4, 125.9, 110.9, 107.0, 96.9, 61.1, 47.1, 32.4, 21.2, 14.1 ppm; IR (neat) $\tilde{\nu}_{max}$ = 1720, 1607, 1580, 1360, 1200, 1130 cm^{-1}; HRMS (ESI): *m/z* calcd for $C_{27}H_{29}N_6O_6^+$ $[M+H]^+$: 533.2141; found: 533.2149.

Dipropyl 3,3′-({1-[2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl]-3-oxo-3*H*-phenoxazin-7-yl}azanediyl)(*E*)-dipropionate (10c)

Compound **6c** (29 mg, 0.049 mmol, 1.0 equiv), **9** (67 mg, 0.296 mmol, 6.0 equiv), [Pd_2(dba)$_3$] (4.5 mg, 0.005 mmol, 0.1 equiv), QPhos (3.5 mg, 0.005 mmol, 0.1 equiv), and KOAc (39 mg, 0.395 mmol, 8.0 equiv) were suspended in 1,4-dioxane (2 mL). The suspension was stirred for 120 min at 100 °C in a sealed vial, after which time it was cooled to room temperature, the volatile components were removed in vacuo, and CH_2Cl_2 (30 mL) was added. The organic layer was washed with water (3×40 mL), separated, concentrated in vacuo, and purified by semipreparative HPLC (linear gradient elution). For fluorescence measurements, the product was further purified by preparative TLC (CH_2Cl_2/methanol 20:1 v/v%) to give **10c** as a black solid (2.8 mg, 10%). R_f (CH_2Cl_2/MeOH 20:1 v/v): 0.5; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.91 (d, *J* = 16.4 Hz, 1H), 7.77 (d, *J* = 16.5 Hz, 1H), 7.72 (d, *J* = 9.0 Hz, 1H), 7.18 (s, 2H), 6.78 (d, *J* = 7.3 Hz, 1H), 6.54 (s, 1H), 6.38 (s, 1H), 4.08 (t, *J* = 6.6 Hz, 4H), 3.83 (t, *J* = 6.6 Hz, 4H), 3.09 (s, 3H), 2.69 (t, *J* = 6.7 Hz, 4H), 1.71–1.63 (m, 4H), 0.95 ppm (t, *J* = 7.3 Hz, 6H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 185.0, 171.2, 166.6, 164.4, 151.4, 150.5, 146.9, 140.7, 138.3, 134.2, 132.5, 129.1, 126.4, 125.9, 110.9, 107.0, 96.9, 66.7, 47.1, 32.3, 21.9, 21.2, 10.3 ppm; IR (neat) $\tilde{\nu}_{max}$ = 2922, 1726, 1609, 1585, 1395, 1360, 1186, 1153, 1130 cm^{-1}; HRMS (ESI): *m/z* calcd for $C_{29}H_{33}N_6O_6^+$ $[M+H]^+$: 561.2456; found: 561.2462.

Cell culture and transfections

For labeling experiments, HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, high glucose) supplemented with 10% FBS (Sigma), 1% L-glutamine (Invitrogen, Palo Alto, USA), 1% sodium pyruvate (Invitrogen), and 1% penicillin–streptomycin (Invitrogen) in a 5% CO_2 atmosphere at 37 °C. Cells were passaged at 80% confluency every 2–4 days for up to 20 passages. For imaging, cells were seeded on 24-well glass-bottomed plates (Sensoplate, Greiner Bio-One) at least 16 h prior to transfection. Prior to cell seeding, 24-well plates were coated with poly-L-lysine hydrobromide (Sigma) for 4–8 h at room temperature. Transfections with the previously reported plasmids ($GFPY^{39TAG}$ and mammalian $PylRS^{AF}$/$tRNA^{Pyl}$ expression plasmids) were performed with JetPrime reagent (PeqLab, Erlangen, Germany), as previously described.[17,18] TCO*-lysine stock solution was prepared in 0.2 M NaOH containing DMSO (15% v/v) at 100 mM concentration. We used TCO*-lysine at a final concentration of 250 μM by diluting the stock solution 1:4 v/v% in 1 M HEPES before adding noncanonical amino acids (ncAAs) to the medium.[18] After 8 h of incubation with ncAAs, cells were rinsed with fresh medium and kept overnight in growth medium without ncAAs.

In vivo labeling of GFP and microscopy

In the morning following transfections, the medium was again exchanged for fresh complete growth medium, with which all subsequent washing and labeling steps were also performed. The transfected cells were then labeled according to a previously described method.[18] Briefly, transfected and non-transfected cells were incubated with a 5 μM solution of each phenoxazine dye (**8a–c**, **10a–c**, diluted from 5 mM DMSO stock solutions stored at −20 °C) dissolved in DMEM for 1 h at 37 °C, and then washed with fresh medium and kept at 37 °C for 1 h before fixation. Fixation was performed with 2% paraformaldehyde (PFA) in PBS at room temperature for 10 min, after the step in which cells were rinsed with PBS. After fixation, cells were kept in PBS and shortly after, taken to the microscope. Confocal imaging was performed on a commercial LEICA TCS SP8 microscope equipped with HC PL APO CS2 63×/1.40 oil objective (Mannheim, Germany). The same acquisition settings were used for all experiments. Cells were imaged in PBS and single-plane images were acquired in the GFP and phenoxazine channels with a pixel size of 180 nm. For GFP, we used predefined settings of the LEICA TCS SP8 microscope; for phenoxazines, we excited each dye at its excitation maximum.

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