Voltage imaging with a NIR-absorbing phosphine oxide rhodamine voltage reporter

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ABSTRACT: Near infrared (NIR) fluorophores may hold the key for non-invasive optical imaging of deep structures in intact organisms with high spatial and temporal resolution. Yet, developing fluorescent dyes that emit and absorb light at wavelengths greater than 700 nm and that respond to biochemical and biophysical events in living systems remains an outstanding challenge. Here, we report the design, synthesis, and application of NIR-absorbing and -emitting, sulfonated, phosphine-oxide (po) rhodamines for voltage imaging in thick tissue from the central nervous system. We find po-rhodamine based voltage reporters, or poRhoVRs, display NIR excitation and emission profiles at greater than 700 nm, show best-in-class voltage sensitivity (up to 43% ΔF/F per 100 mV in HEK cells), and can be combined with existing optical sensors, like Ca²⁺-sensitive fluorescent proteins (GCaMP), and actuators, like light-activated opsins ChannelRhodopsin-2 (ChR2). Simultaneous voltage and Ca²⁺ imaging reveals differences in activity dynamics in rat hippocampal neurons, and pairing poRhoVR with blue-light based ChR2 affords all-optical electrophysiology. In ex vivo retinas isolated from a mouse model of retinal degeneration, poRhoVR, together with GCaMP-based Ca²⁺ imaging and traditional multi-electrode array (MEA) recording, can provide a comprehensive physiological activity profile of neuronal activity. Taken together, these experiments establish that poRhoVR will open new horizons in optical interrogation of cellular and neuronal physiology in intact systems.

INTRODUCTION

Fluorescence microscopy has revolutionized the life sciences. Advances in both synthetic and genetically encoded fluorophores make fluorescence microscopy routine in laboratories across the globe. Coupled with progress in areas like super-resolution microscopy,¹² light sheet microscopy,³ and tissue clearing,⁴,⁶ fluorescent dyes bring the high spatial and temporal resolution of optical microscopy to bear on samples ranging from single molecules to interacting cells.

However, optical imaging in thick tissue and intact organisms remains challenging. Commonly used fluorophores, like fluorescein and rhodamine, possess excitation and emission profiles within the visible region of the spectrum and overlap extensively with endogenous chromophores, especially heme.⁷⁻⁸ Although alternative imaging modalities like magnetic resonance imaging, positron emission tomography, and photoacoustic imaging afford excellent penetration into thick tissue, these modalities require specialized instrumentation, are half-life limited, require radioactive reagents, and/or cannot access the micrometer spatial and millisecond time resolution routinely afforded by optical microscopy.⁹

In the context of neurobiology, an outstanding challenge is tracking neuronal voltage dynamics with sub-micron spatial resolution and sub-millisecond temporal resolution within intact tissues. Although two-photon (2P) microscopy provides access to structures in deeper tissue, traditional raster-scanning 2P microscopy cannot achieve the temporal resolution required to image large numbers of neurons simultaneously.

Therefore, fluorescent dyes and voltage indicators that possess near-infrared (NIR, 700 – 1000 nm)¹⁰⁻¹⁴ excitation and emission profiles are of considerable interest because they promise to bridge the gap between modalities with good tissue penetration but poor resolution and optical microscopy, with excellent spatial resolution, but poor tissue penetration. First, NIR photons scatter less in thick tissue. Second, by avoiding endogenous chromophores like heme (<650 nm) and water (>900 nm),⁷,⁸,¹⁵ fluorophores that operate in the so-called NIR window gain profound advantages for tissue and in vivo imaging. Finally, NIR fluorophores can be deployed for multiplex imaging alongside existing fluorescent labels, indicators, and actuators that use visible wavelengths of light.

Despite the attraction of NIR dyes that both absorb and emit at wavelengths above 700 nm, traditionally-employed NIR dyes, such as porphyrins and phthalocyanins,¹⁶ polymethines,¹⁰ BODIPY derivatives,¹⁶⁻¹⁸ and xanthenes with extended annulation¹⁹⁻²¹ tend to suffer from high molecular weights, high hydrophobicities, low water solubility, a propensity to aggregate, and chemical or photochemical instability, complicating their use in biological contexts. A number of creative approaches have
recently been employed to address these concerns.\textsuperscript{14, 20, 22-24} In particular, xanthene-based dyes are attractive because the compact, fused 3-ring system promotes stability and decreases molecular weight. Annulation of xanthenes can push their absorbance into ranges >700 nm,\textsuperscript{19} at the expense of decreased water solubility. More recent efforts focus on replacing oxygen with carbon\textsuperscript{25-27} or other heteroatoms such as S\textsuperscript{28-30} and P. Indeed, recent examples of phosphinate\textsuperscript{31} and phosphoxine oxide based xanthene dyes\textsuperscript{32-33} demonstrate this unique scaffold can access absorption profiles at or above 700 nm while maintaining a compact, three-ring structure. While numerous approaches to NIR-emitting fluorophores exist, there are relatively few examples of NIR-absorbing fluorescent indicators—fluorophores that sense and respond reversibly to changes in local environment. NIR-absorbing indicators tend to employ polymethine, rather than xanthene, scaffolds.\textsuperscript{3, 34-36}

Therefore, we were eager to adapt phosphorous-substituted xanthenes into a fluorescent sensing scaffold. Previous work in our lab suggests that xanthene dyes with a range of bridgehead (O,\textsuperscript{37} C,\textsuperscript{37} Si\textsuperscript{38}) and terminal atoms (O\textsuperscript{27, 37}, or substituted N\textsuperscript{38-39}) can be employed as voltage-sensitive dyes via introduction of a lipophilic, conjugated molecular wire. We hypothesized that the installation of phenylevinylene molecular wires into the context of a phosphorous-substituted xanthene dye would yield voltage-sensitive indicators with emission and excitation maxima above 700 nm. This would fill an important void: although there are several examples of chemically-synthesized\textsuperscript{40-42} and genetically encoded\textsuperscript{43-44} voltage indicators with emission maxima beyond 700 nm, to date, few examples exist of voltage sensitive dyes with peak excitation greater than 700 nm.\textsuperscript{46-47}

We now present the phosphine-oxide Rhodamine Voltage Reporters, or poRhoVRs, which all feature excitation and emission maxima above 700 nm, up to 43% ΔF/F per 100 mV voltage sensitivity, compatibility with commonly used optical sensors and actuators, including Oregon Green BAPTA (OGB), GCaMP6, and ChannelRhodopsin-2 (ChR2), and the ability to report on neuronal activity in retinal tissue isolated from a mouse model of retinopathy.

Results

Synthesis of sulfonated phosphorous rhodamines

Although phosphorous rhodamines possess attractive optical properties\textsuperscript{31-33}—NIR excitation and emission, photostability, and good water solubility—their tendency to localize to intracellular membranes, including mitochondria\textsuperscript{35} and lysosomes,\textsuperscript{48} precludes their straightforward deployment as cytosolic indicators or voltage sensors. Water solubility and exclusion from cellular uptake can be achieved through installation of anionic groups on the exocyclic rhodamine nitrogens;\textsuperscript{49-51} however, we recently found that sulfonation of the meso aromatic ring of rhodamine-type systems prevents cellular uptake of dyes.\textsuperscript{38, 51} We hypothesized that a similar strategy could provide sulfonated phosphorous rhodamines for incorporation into voltage sensing scaffolds.

Phosphine-oxide RhoVRs were accessed in 6 steps from commercially available triphenylphosphonium salts (Scheme 1). Anilino phosphate oxide 4 was prepared in high yield over three steps.\textsuperscript{32, 52} Phosphate oxide 4 was alkylated through a slightly modified procedure using ethyl iodide and potassium carbonate in dimethyformamide (DMF) to produce phosphate oxide 5 in 89% yield. The key sulfonated phosphorous rhodamine precursors with bromine substitution for installation of the phenylevinylene molecular wire were prepared in one step by condensing bromosulfobenzaldehydes\textsuperscript{51} or with phosphate oxide 5 in the presence of urea\textsuperscript{53} and glacial acetic acid (Scheme 1). Other attempts at condensation with methane sulfonic acid or propionic acid without urea resulted in no reaction or were extremely low yielding.

The condensation between m- (6) or p-bromosulfobenzaldehyde (7) and phosphate oxide 5 produced two isomers of m- (8) and p- (9) bromosulfophosphate oxide rhodamines\textsuperscript{32-33} in which the P-methyl substituent and sulfonate exist in either a cis or trans relationship to one another (Figure 1b), consistent with previous reports and nomenclature\textsuperscript{52} (Figure S1). For both 8 and 9, the two isomers were separable on a basic alumina column, eluting with 0-5% methanol in dichloromethane. After this step, no further purification was necessary for the major isomer of either 8 or 9. Preparative HPLC was required to isolate the minor isomer. Analysis of the NMR spectra of the purified major and minor isomers of 8 and 9 revealed differences in the chemical shifts of both the phosphorous atom and the P-methyl substituents (Table S1): for the P-methyl group, δ = 2.00 ppm (\textsuperscript{1}H, major isomer) and δ = 1.96 (and Figure S1). Furthermore, x-ray crystal structures of the major isomer of both 8 and 9 reveal a cis relationship between the sulfonate and the methyl group on the phosphorous bridgehead atom (Figure 1c and d).

Attempts to crystallize the minor isomer of 8 or 9 were unfruitful. In aqueous solution (phosphate buffered saline, 5 μM dye), both bromo phosphate oxide rhodamine 8 – cis and 9 – cis had an absorption
maximum at 704 nm, emission maxima around 728 nm (Figure 1a), and quantum yields near 20% (22% for 8 and 19% for 9).

Synthesis of phosphorous rhodamine voltage reporters

After determining that sulfonated po-rhodamines have reasonable quantum yields and desirable NIR excitation and emission maxima above 700 nm, we hypothesized that incorporation of phenylene vinylene molecular wires should render these NIR emitters maximal at 700 nm, and emission maxima above 700 nm. We investigated the synthesis of phosphorous rhodamine dyes 8 and 9. a) Plots of normalized absorbance (solid lines) and fluorescence intensity (dashed lines; excitation provided at 625 nm) of phosphine-oxide rhodamine 8 (blue lines) and 9 (black lines) in phosphate buffered saline (5 μM dye, 1% DMSO). b) Chemical structure of the cis isomers of 8 and 9. c) Hydrogen atoms, lattice solvent molecules, and resolved disordered fragments have been omitted for clarity.

Figure 1. Characterization of sulfonated phosphine-oxide rhodamine dyes 8 and 9. a) Plots of normalized absorbance (solid lines) and fluorescence intensity (dashed lines; excitation provided at 625 nm) of phosphine-oxide rhodamine 8 (blue lines) and 9 (black lines) in phosphate buffered saline (5 μM dye, 1% DMSO). b) Chemical structure of the cis isomers of 8 and 9. c) Hydrogen atoms, lattice solvent molecules, and resolved disordered fragments have been omitted for clarity.

Cellular characterization of poRhoVR indicators in HEK cells. (a-d) Widefield, epifluorescence images of poRhoVR 14 (1 μM) in HEK cells. Cells are counter-stained with b) rhodamine 123 (1 μM) and c) Hoechst 33342 (1 μM) to visualize mitochondria and nuclei, respectively. d) An overlay of poRhoVR 14, rhodamine 123, and Hoechst 33342. Scale bar for (a-d) is 20 μm. e) Plot of fractional change in fluorescence of poRhoVR 14 vs time for 40 ms hyper- and depolarizing voltage steps from a holding potential of -60 mV for a single HEK cell labeled with poRhoVR 1 (14, 1 μM). f) Plot of ΔF/F vs membrane potential, summarizing data from n = 6 individual HEK cells. Error bars are ± S.D. If error bars are not visible, the error is smaller than the marker.

All of the poRhoVR compounds possess excitation and emission profiles well-matched to the parent phosphine-oxide rhodamines: absorption was maximal at 700 nm, and emission peaked at around 728 nm (Table 1, Figure S3a-d)

Cellular characterization of phosphorous rhodamine voltage reporters

All four poRhoVR dyes (12 – 15) localize to the cellular membrane and display differing cellular brightness when applied to HEK cells under identical experimental conditions (Figure S2a-d). Although all poRhoVRs localize to the membrane, poRhoVRs 13 and 15, with N,N-diethyl, methoxy-aniline substituents, are approximately 10-fold dimmer in cell membranes than poRhoVR 12 and 14 (N,N-dimethyl aniline) (Table 1, Figure S2e). The long wavelength excitation and emission of poRhoVRs enables multi-color imaging with organelle specific dyes. Live-cell imaging of poRhoVR 14 (1 μM, Figure 2a) in HEK cells co-stained with rhodamine 123 (mitochondria, Figure 2b) and Hoechst 33342 (nuclei, Figure 2c) demonstrate good membrane localization for poRhoVRs along with compatibility with simultaneous, multi-color imaging (Figure 2d).

The cellular fluorescence of poRhoVRs is voltage sensitive. Simultaneous fluorescence microscopy during sequential depolarizing and hyperpolarizing steps from a holding potential of -60 mV in HEK cells under whole-cell voltage-clamp conditions reveals linear fluorescence vs. voltage relationships for poRhoVR dyes 12 – 14 (Figure 2e and f, 14; Figure S3 for all indicators). Voltage sensitivities range from 13% (poRhoVR 12) to 43% (poRhoVR 13) per 100 mV (Table 1). The sensitivity of poRhoVR 13, at 43%, is nearly 2-fold the sensitivity of our most red-shifted voltage indicator to date, the Si-rhodamine based BeRST (24% ΔF/F per 100 mV) and is comparable to...
Figure 3. Voltage imaging in dissociated rat hippocampal neurons with poRhoVR 14. Transmitted light image of neurons loaded with a) poRhoVR 14 (500 nM). b) Epifluorescence image of neurons showing poRhoVR 14 staining. Scale bars are 20 μm. c) Plot of fractional change in poRhoVR 14 fluorescence (ΔF/F) vs time emanating from cells 1-4 in image (b). Optical sampling rate is 500 Hz. Asterisks indicate subthreshold voltage changes.

The sensitivity of our previously reported, tetra-methyl rhodamine based RhoVR (47% ΔF/F per 100 mV), however, the low cellular brightness of poRhoVR 13 is approximately 8 to 12-fold brighter than 13 under identical experimental conditions (Figure S5a). In a complementary fashion, the ΔF/F value per spike/action potential for 13 was larger (12% vs 6%, Figure S5b) but with lower overall signal-to-noise ratio (SNR, 20:1 vs 40:1, Figure S5c). Because of the higher brightness and SNR for detecting action potentials, we used poRhoVR 14 for subsequent experiments.

poRhoVR 14 and optogenetic activator ChR2
	not only for tracking static fluorescence associated with distinct organelles (Figure 2a-d), but also in concert with commonly employed optogenetic actuators and sensors. We expressed the blue light-activated opsin, Channelrhodopsin-2 (ChR2), fused to yellow fluorescent protein (YFP) in a subset of hippocampal neurons isolated from rat (Figure 4a and b). Bath application of poRhoVR 14 to these same neurons results in membrane-localized fluorescence (Figure 4c) that is spectrally isolated from the YFP signal (Figure 4d).

The combined use of ChR and poRhoVR allows dissection...
of functional connectivity across a large number of neurons. Optical stimulation of cell 1 (475 nm, 5 ms, 1.92 mW/mm²) results in ChR2-evoked action potentials optically recorded at cell 1 (Figure 4c and f). Cells 4 and 5 appear monosynaptically coupled to ChR2-positive cell 1 with action potential latencies of 6.7 ms ± 1.5 ms (S.D., n = 60 pairs of spikes) and 5.6 ms ± 1.3 ms (S.D., n = 20 out of 60 spikes), respectively. However, the data reveals differences in the relative strengths of these connections with cell 1 triggering firing in cell 4 for 100% of action potentials, and only 33% for cell 5. Interestingly, bursts of spontaneous activity indicate strong recurrent connectivity between all neurons imaged, but firing initiated in cell 1 activates only a subset of these neurons.

The combination of poRhoVR and ChR2 enables interrogation of sub-threshold potentials. In a few cases, cyan light-evoked ChR2 activity in cell 1 results in sub-threshold, postsynaptic potentials in cells up to 400 microns away (Figure 5d and e, cells 2 – 4, asterisks), highlighting the ability of poRhoVR 14 to monitor sub-threshold voltage changes (Figure 3a–c). The use of cyan light to stimulate ChR2 does not cross-excite poRhoVR 14, as indicated by the lack of stimulus artifact in the ChR2-negative cells in the same field of view (Figure 4d and e, Figure 5d). Together, these experiments establish poRhoVR 14 as a powerful complement for all-optical electrophysiology utilizing NIR absorbing indicators.

Two color imaging with poRhoVR 14 and Ca²⁺ indicators

In addition to deployment alongside light-activated actuators, poRhoVR can be used with optical indicators. Fluorescent sensors for Ca²⁺ are among the most widely used optical sensors. Despite some three decades since the initial reports of fluorescent indicators for this critical intracellular messenger, most Ca²⁺ indicators utilize excitation and emission profiles firmly centered in the blue/green region of the visible spectrum (for example, Oregon Green BAPTA, OGB, and the GCaMP family of genetically encoded indicators). Although promising new Ca²⁺ indicators, both synthetic and genetically encoded, possess red-shifted excitation and emission spectra, circularly-permuted (cp) GFP-based indicators, like the GCaMP family, dominate the landscape of functional imaging. Therefore, fluorescent voltage indicators with orthogonal wavelengths are required.

We performed two-color, simultaneous voltage and Ca²⁺ imaging in the same cells using poRhoVR 14 and the synthetic Ca²⁺ indicator, OGB (Figure 5a–c). We treated hippocampal neurons with both poRhoVR 14 (500 nM) and OGB (1 μM) simultaneously and imaged using an image-splitting device to project two emission wavelengths onto the same camera chip. Under these conditions, we observe clear membrane-associated fluorescence for poRhoVR 14 (Figure 5a) and cytosolic fluorescence for OGB (Figure 5b). We established that no cross-excitation exists under these conditions (Figure S7). Using field-stimulation electrodes, we evoked a series of 10 action potentials, across a range of frequencies, and simultaneously recorded voltage (Figure 5c, magenta traces) and Ca²⁺ (Figure 5c, green traces) dynamics.

Both poRhoVR 14 and OGB clearly resolve single action potentials when activity is evoked at rates of either 5 or 10 Hz. poRhoVR 14 clearly resolves action potentials at firing rates of 20 and 30 Hz (Figure 5c). OGB, despite its fast Ca²⁺ response kinetics (< 5 ms to action potential peak) compared to GCaMP6f (~45 ms to peak) and other genetically encoded indicators, fails to accurately report individual action potential-evoked Ca²⁺ transients at firing frequencies higher than 10 Hz (Figure 5c). Neurons in the brain and retina can fire action potentials at rates up to several hundred Hz, for example in interneurons of the hippocampus, Purkinje cells of the cerebellum, and ganglion cells of the retina, emphasizing the need for indicators with fast response kinetics.

Ca²⁺ indicators are often characterized against varying numbers of action potentials arriving at a constant frequency. However, neural information is often encoded in the form of spike rates, resolution of individual spikes and firing frequency is critical for understanding the underlying physiology of the system.

Figure 5. Simultaneous voltage and calcium imaging with poRhoVR 14. Epifluorescence image of neurons stained with both a) poRhoVR 14 (1 μM) and b) Oregon Green BAPTA 1 AM (OGB, 1 μM). c) Plots of ΔF/ΔF for voltage (poRhoVR 14, purple) and Ca²⁺ transients (OGB, green) in response to field stimulation driven at 5, 10, 20, and 30 Hz. d) Epifluorescence image of a neuron transfected with GCaMP6s. e) This same GCaMP6s (+) neuron is also stained with poRhoVR 14 and imaged simultaneously. Scale bar is 10 μm. f) Simultaneously recorded traces of voltage and calcium activity from neuron in panels d and e. Activity was evoked using field stimulation at a rate of 16 Hz. g) The insets show that the onset and decay of voltage signals imaged with poRhoVR 14 precede that of the calcium signal visualized from the same cell with GCaMP6s.
under observation. Even with very fast OGB, estimating spike frequency using Ca$_{2+}$ imaging traces alone was unsuccessful. Neither peak Ca$_{2+}$ ΔF/F (Figure S5a) nor integrated area under the curve (Figure S8b) were able to resolve differences at 5, 10, 20, or 30 Hz firing rates. In contrast, the optically recorded voltage transients revealed by poRhoVR 14 clearly discriminates between firing frequencies of 5, 10, 20, and 30 Hz (Figure S8c).

Simultaneous voltage and Ca$_{2+}$ imaging in the same cells can also be achieved alongside genetically encoded indicators, like GCaMP6. We again stained neurons with poRhoVR 14 (1 μM). This time, a subset of hippocampal neurons expressed GCaMP6s. Again, poRhoVR localizes to membranes (Figure 5d), while GCaMP6s fluorescence appears cytosolic (Figure 5e). Simultaneous voltage and Ca$_{2+}$ imaging of spontaneous activity in hippocampal neurons reveals fast-spiking bursts resolved in voltage (Figure 5f-h, magenta trace), followed by slower, sustained increases in GCaMP6-associated fluorescence (Figure 5f-h, green trace). Notably, voltage imaging with poRhoVR 14 exhibits sufficiently high temporal resolution to distinguish individual action potentials in spike volleys (Figure 5g, 8 spikes; Figure 5h, 9 spikes), while Ca$_{2+}$ imaging does not. Together, these experiments establish the utility of poRhoVR dyes for monitoring fast spiking in neurons alongside commonly used synthetic and genetically-encoded Ca$_{2+}$ indicators.

**Voltage and Ca2+ imaging and electrode recording in a mouse model of retina degeneration**

The NIR (>700 nm) excitation and emission spectra of poRhoVR dyes, along with their good voltage sensitivity and compatibility with commonly used optogenetic sensors and actuators, makes poRhoVR 14 a promising candidate for mapping voltage dynamics in thick, visible-light sensitive neural tissue like retinas. The retina is a highly organized and accessible out-post of the central nervous system. Light responses initiated in rods and cones are synchronically transmitted to bipolar cells, which activate retinal ganglion cells (RGCs). RGCs generate the action potentials that carry visual information to the brain. In normally functioning retinas, the intrinsic light sensitivity of photoreceptors in rods and cones complicates optical imaging of both voltage and Ca$_{2+}$ transients in RGCs. We applied poRhoVR to investigate membrane potential dynamics in retinas from a mouse model of retina degeneration. In particular, retinas from rd1 mice are an attractive model system in which functional imaging can be applied to explore mechanisms underlying certain genetic forms of blindness and retinal degeneration.

The rd1 mouse is a commonly employed to study retinal degeneration. Lacking a functional β subunit of rod cGMP phosphodiesterase (βPDE), these mice suffer rapid loss of rod
cells, followed by loss of cone cells. As a result, these mice lack rods and cones and are therefore blind, yet still retain functional RGCs in the ganglion cell layer (GCL). Therefore, apart from the rare melanopsin-expressing, intrinsically sensitive RGC (<2% of the RGCs) the surviving RGCs in rd1 retinas have no light response, but remain spontaneously active, and continue to receive synaptic input from bipolar cells.

We prepared ex vivo, flat-mount retinas from rd1 mice that express GCaMP6f in retinal ganglion cells (RGCs). Bath application of poRhoVR 14 (5 μM, in oxygenated ACSF) results in diffuse poRhoVR staining throughout the tissue, as assessed by widefield fluorescence microscopy (Figure 6a, Figure S9). In retinas from rd1 mice, we observed spontaneous oscillations of poRhoVR fluorescence (Figure 6d-h, magenta traces). To confirm that these changes in poRhoVR dynamics correspond to voltage changes in RGC, we paired voltage imaging with multi-electrode array (MEA) of extracellular potential.

The poRhoVR-stained retinas were mounted on a 64-channel MEA to simultaneously record transmembrane potential (poRhoVR), extracellular potential (MEA), and Ca2+ transients (GCaMP6f) in many RGCs. Simultaneous transmembrane voltage imaging and MEA recording of extracellular potentials confirms that poRhoVR readily reports on neuronal activity within RGCs of the retina (Figure 6c-f). Voltage imaging reveals spontaneous membrane potential depolarizations that appear clustered in bursts throughout the imaging session (Figure 6c and d, magenta) These optically-recorded oscillatory bursts are, to our knowledge, the first direct imaging of membrane potential dynamics in rd1 retinas, and are consistent with previous MEA recordings in rd1 retinas.79-80 GCaMP6f recordings from the same areas revealed slower, Ca2+ transients that were delayed relative to increases in poRhoVR fluorescence (Figure 6c and d, green), similar to simultaneous Ca2+ / voltage imaging in hippocampal neurons.

Voltage imaging with poRhoVR enables dissection of the temporal evolution of neuronal activity in rd1 retina. Synaptic isolation of RGCs via blockade of all major forms of excitatory and inhibitory synaptic transmission results in evolution of activity from short, unsynchronized firing patterns (Figure 6c, d, and g) to synchronous and sustained firing (Figure 6e, f, and h). Voltage imaging with poRhoVR also allows investigation of the spatial differences in voltage dynamics with the retina. We record fluorescence from a region of interest (ROI) composed of a ring with a width approximately matching that of an RGC (~10 μm) around the MEA electrode (Figure 6a and b). This enables a direct comparison to extracellular potentials recorded by the MEA (Figure 6c and d, black). For the two electrodes visible in the recording image (Figure 6a and b), the MEA recording is closely matched by the poRhoVR dynamics (Figure 6c and d, black vs. magenta). The use of poRhoVR allows examination of the spatial relationships of voltage changes at different electrodes (Figure S9). poRhoVR signals recorded in ROIs close to an electrode strongly resemble the MEA recording at that electrode, while poRhoVR dynamics further from electrode present as a convolution of the two MEA recordings (Figure S9).

Conclusion

In summary, we present the design, synthesis, and application of phosphine-oxide rhodamines for voltage imaging. These new poRhoVRs have excitation and emission profiles above 700 nm and possess good voltage sensitivity. Their compatibility with other optical sensors and actuators makes them a powerful complement to existing approaches to dissect neuronal activity. We show that poRhoVR 14 can report on spontaneous action potentials in rat hippocampal neurons and enables all-optical electrophysiological manipulations with ChR2. poRhoVR can be deployed alongside multi-electrode arrays and Ca2+ imaging to record from many RGCs at once in the intact retina of a mouse model of retinal degeneration. The ability to access NIR wavelengths for both excitation and emission enable imaging over large areas in opaque tissues at acquisition speeds that would be impossible achieve with traditional two-photon raster scanning techniques.

ASSOCIATED CONTENT

Supplementary data, including supporting figures, spectra, procedures, and analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting Information for

Voltage imaging with a NIR-absorbing phosphine oxide rhodamine voltage reporter

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Table S1. Tabulated Spectroscopic Properties of poRhoVR and poRhodamine isomers

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Figure S1. Chemical characterization of isomers of phosphine oxide rhodamine dyes.

Figure S1. Chemical characterization of isomers of phosphine oxide rhodamine dyes. (center) Decoupled \(^{31}\text{P}\) spectra the trans- and cis- isomers of po-rhodamines 8 and 9 and for poRhoVR 13. (sides) Proposed structures of 8, 9, and 13. The structure of 8 – cis and 9 – cis were confirmed by x-ray crystallography (Figure 1b and c). The structure of the major isomer of 13 is assumed to be the cis isomer, based on similarities in the \(^{31}\text{P}\) NMR. \(^{1}\text{H}\) labels on the structures (a, b, and c) refer to the indicated chemical shifts in Table S1.
Figure S2. Quantification of poRhoVR staining in HEK cells.

(a-d) Widefield epifluorescence images of poRhoVR indicators in HEK cell; a) poRhoVR 12, b) poRhoVR 13, c) poRhoVR 14, and d) poRhoVR 15. Scale bar for (a-d) is 20 μm. 

(e) Plot of fluorescence intensity of HEK cells stained with 2 μM poRhoVR dyes (12 – 15). Bars represent mean fluorescence intensity for n = 3 independent coverslips of HEK cells. The average value for a coverslip of HEK cells stained with poRhoVR is shown as a blue dot. Each coverslip contained 30 to 60 individual cells/regions of interest (ROIs) that were used to determine the mean fluorescence. Error bars depict standard deviation (n = 3).
Figure S3. Absorption/Emission Spectra and Voltage sensitivity of poRhoVR dyes.

Plot of normalized absorption (purple) and emission (blue) for poRhoVR dyes a) 14, b) 12, c) 15, and d) 13. Spectra were acquired at 1 μM (0.1 % DMSO) in PBS buffer. For emission spectrum, excitation was provided at 625 nm.

Voltage sensitivity plots for poRhoVR dyes e) 14, f) 12, g) 15, and h) 13. Upper row depicts plots of ΔF/F vs. final membrane potential (mV) in HEK cells loaded with 1 μM poRhoVR dye and subjected to whole-cell voltage-clamp conditions. Error bars represent standard deviation for n = 6, 5, 5, and 4 cells, respectively. Lower row depicts plots of ΔF/F (%) vs time for HEK cells loaded with 1 μM poRhoVR and held at -60 mV under whole-cell voltage-clamp and stepped to hyper- and depolarizing potentials in increments of 20 mV (±100 mV).
Figure S4. Voltage imaging in hippocampal neurons with poRhoVR 13.

Transmitted light image of neurons loaded with a) poRhoVR 13 (500 nM). b) Epifluorescence image of neurons showing poRhoVR 13 staining. Scale bars are 20 μm. c) Plot of fractional change in poRhoVR 13 fluorescence (ΔF/F) vs time emanating from cells 1-4 in image (b). Optical sampling rate is 500 Hz.
Figure S5. Quantification of brightness, voltage sensitivity and signal to noise of selected poRhoVR dyes in neurons

Plot a) fluorescence intensity, b) fractional change in fluorescence in response to evoked action potentials (ΔF/F), and c) signal-to-noise ratios (SNR) for these action potentials vs. illumination intensity in neurons. Hippocampal rat neurons were stained with poRhoVR 13 (grey) or 14 (red) at concentrations of 500 nM (light red/grey), 1 μM (red/grey), or 2 μM (dark red/dark grey) poRhoVR dye and imaged at 500 Hz. Data represent mean values ± S.E.M. for n = 4 images, comprising approximately 10-20 neurons per image.
Figure S6. Compatibility of poRhoVR 14 with ChR2 stimulation

a) Transmitted light image of dissociated rat hippocampal neurons stained with poRhoVR 14 (500 nM). Scale bar is 20 μm.
b) Epifluorescence image of neuron displaying YFP genetic marker for ChR2.
c) Epifluorescence image of neurons stained with poRhoVR 14.
d) Recording of ΔF/F from the cell bodies of neurons indicated in panels a-c. e) Expanded time base recording of activity within the red, dashed box in panel d. Optical sampling rate was 500 Hz. ChR2 (+) cell 1 was stimulated optically with flashes of cyan light (475 nm, 5 ms, 1.92 mW/mm²) as indicated by the cyan bars below the blue optical recording in panel d. Notably, there is no visible cyan light excitation of poRhoVR 14 (see for example, ΔF/F plot of cell 6, in panels d and e).
Figure S7. Compatibility of poRhoVR 14 with OGB Ca\textsuperscript{2+} imaging in rat hippocampal neurons. 25% cyan illumination; no red illumination

Figure S7. Compatibility of poRhoVR 14 with OGB Ca\textsuperscript{2+} imaging in rat hippocampal neurons. Rat hippocampal neurons with stained with poRhoVR 14 and OGB and imaged using an image-splitter to enable acquisition of simultaneous NIR (poRhoVR) and green (OGB) fluorescence, as in Figure 5 (main text). a-d) Plots of fluorescence intensity vs. time for OGB (green traces) and poRhoVR 14 (magenta traces) vs. time (ms) with cyan illumination only (17.6 mW/mm\textsuperscript{2}). Under single wavelength (cyan) illumination, field stimulation was provided at a) 5, b) 10, c) 20, and d) 30 Hz. e-h) Plots of fluorescence intensity vs. time for poRhoVR 14 (magenta traces) and OGB (green traces) vs. time with red illumination only (50%, 49.7 mW/mm\textsuperscript{2}). Under single wavelength (red) illumination, field stimulation was provided at e) 5, f) 10, g) 20, and h) 30 Hz. Each plot shows the responses of two neurons (recorded simultaneously in red and green channels).
Figure S8. Quantification of evoked activity in neurons using poRhoVR 14 and Oregon Green BAPTA

Calcium Imaging (OGB)                      Voltage Imaging (poRhoVR)

Figure S8. Quantification of evoked activity in neurons using poRhoVR 14 and Oregon Green BAPTA. Analysis of data presented in Figure 5 of main text. Ca$^{2+}$ imaging data with OGB was quantified as a) peak $\Delta F/F$ or b) integrated area under the curve per second vs stimulus frequency, in Hz. For Ca$^{2+}$ imaging data, black bars represent the mean ± S.E.M. for $n = 40$ cells. Data points represent individual cells. There are no statistically-significant differences between stimulus frequency when analyzing peak $\Delta F/F$ or AUC per second (one-way ANOVA + Tukeys comparisons). Voltage imaging data with poRhoVR 14 was quantified as c) the mean instantaneous frequency (Mean Inst. Frequency), in Hz, vs stimulus frequency, in Hz. For voltage imaging data, black bars represent the mean ± S.E.M. for $n = 40$ cells. Data points represent the mean instantaneous frequency between spikes for each analyzed cell (10 spikes, 9 intra-spike intervals). For voltage imaging, all of the differences between stimulus frequencies are significant ($p < 0.0001$, one-way ANOVA Kruskal-Wallis test + Dunns comparison).
Figure S9. Spatial evolution of voltage signals in rd1 mouse retina, monitored by poRhoVR. 

- **Figure S9.** Spatial evolution of voltage signals in rd1 mouse retina, monitored by poRhoVR. 
  - **a)** Widefield, epifluorescence image of rd1 mouse retina stained with poRhoVR 14. Black circles are the electrodes, labeled “e25” and “e26”. Rectangles indicate regions of interest. Plots of relative change in fluorescence (arbitrary units) from either 
  - **b)** poRhoVR 14 or 
  - **c)** GCaMP6 vs time for the ROIs indicated in panel (a). Higher resolution, widefield, epifluorescence image of rd1 mouse retina stained with 
  - **d)** poRhoVR 14 and expressing 
  - **e)** GCaMP6.
Supporting Information Methods

HEK293T cell culture information

HEK293T cells were obtained from the UC Berkeley Cell Culture Facility. Cells were passaged and plated onto 12 mm glass coverslips coated with Poly-D-Lysine (PDL; 1 mg/mL; Sigma-Aldrich) to a confluency of approximately 15% and 50% for electrophysiology and imaging, respectively. HEK293T cells were plated and maintained in Dulbecco’s modified eagle medium (DMEM) supplemented with 4.5 g/L D-glucose, 10% fetal bovine serum (FBS), and 1% Glutamax. Cells were maintained at 37 °C in a humidified incubator with 5 % CO₂.

Preparation of primary neuron cultures

Hippocampi were dissected from embryonic day 19 Sprague Dawley rats (Charles River Laboratory) in cold sterile HBSS (zero Ca²⁺, zero Mg²⁺). Hippocampal tissue was treated with trypsin (2.5%) for 15 min at 37 °C. The tissue was triturated using fire polished Pasteur pipettes, in minimum essential media (MEM) supplemented with 5% FBS, 2% B-27, 2% 1M dextrose and 1% GlutaMax. The dissociated cells were plated onto 12 mm diameter coverslips (Fisher Scientific) pre-treated with PDL at a density of 25,000-30,000 cells per coverslip in MEM supplemented media (as above). Neurons were maintained at 37 °C in a humidified incubator with 5 % CO₂. After 1 day in vitro (DIV) half of the MEM supplemented media was removed and replaced with Neurobasal media containing 2% B-27 supplement and 1% GlutaMax. Functional imaging was performed on mature neurons 13-20 DIV.

General Imaging Parameters

Imaging experiments were performed on either an upright or an inverted epifluorescence microscope, AxioExaminer Z-1 (Zeiss), equipped with a Spectra-X Light engine LED light (Lumencor), and an OrcaFlash4.0 sCMOS camera (Hamamatsu). The following LED light sources were used; red (631/28 nm bandpass), teal LED (510/25 nm bandpass), cyan (475/34 nm bandpass), violet (390/22 nm bandpass). The microscope was controlled via Slidebook (v6, Intelligent Imaging Innovations) or MicroManager (Studio Version 1.4.22). Imaging was done with a W-Plan-Apo 20x/1.0 objective (20x; Zeiss) or a W-Plan-Apo 63x/1.0 objective (63x; Zeiss). The QUAD emission filter set, a quadruple dichroic mirror (432/38, 509/22, 586/40, 654 nm LP) combined with a (430/32 nm, 508/14 nm, 586/30 nm, 708/98 nm), was routinely used. For experiments with simultaneous voltage and calcium imaging a Dual View emission splitter (Optical Insights) was used. The Dual-View contained a 585dcxr dichroic and two emission filters (520/28 nm and 723/68 nm) which separated the calcium (GCaMP or Oregon Green BAPTA-AM) and poRhoVR fluorescence signals.

Image Analysis

Analysis of voltage sensitivity in HEK cells was performed using custom MATLAB routines in which a region of interest (ROI) was automatically selected based on changes in fluorescence intensity. The ROI is then applied as a mask to all frames in the image stack. Fluorescence
intensity values were calculated at known baseline and voltage step epochs and used to calculate a percent change in baseline fluorescence (% ΔF/F) per 100 mV change in membrane potential.

Optical traces of neuronal calcium and voltage activity were obtained by drawing ROIs around the cell bodies of neurons, transfected with GCaMP6s or stained with poRhoVR or OGB-AM, in ImageJ and extracting the mean cellular fluorescence from each frame in the image stack. The mean background fluorescence was subtracted from the mean cellular fluorescence of each frame. ΔF/F values were calculated by first subtracting a mean background value from all raw fluorescence frames to give a background subtracted trace (bkgsub). A baseline fluorescence value (F_base) is calculated by averaging the fluorescence of 10 to 20 frames that show no activity. F_base was subtracted from each timepoint of the bkgsub trace to yield a ΔF trace. The ΔF was then divided by F_base to give ΔF/F traces.

A custom ImageJ macro aligned and split the image stack into two, one for the GCaMP6s signal and the other for the poRhoVR 14 signal. Custom MATLAB routines were used to extract raw fluorescence traces of retinal activity. Either ring-shaped ROIs 16 pixels (20.8 µm) wide or a series of 7 rectangular ROIs each 16 pixels (20.8 µm) by 32 pixels (41.6 µm) were drawn around or between the electrodes. These ROIs were applied as masks to all frames in the image stack. poRhoVR 14 traces were bleach corrected by approximating the bleach curve via asymmetric least squares and subtracting the resulting curve from the raw poRhoVR 14 signal. A baseline fluorescence value (F_base) is calculated by averaging the fluorescence of 10 to 20 frames that show no activity. F_base was subtracted from each timepoint of the the bleach corrected poRhoVR 14 traces or the raw GCaMP6s traces to yield a ΔF trace. The ΔF was then divided by F_base to give ΔF/F traces.

**Figure 2. Cellular characterization of pRhoVR indicators in HEK cells**

**Multi-color imaging (Figure 2 a-h)**

HEK293T cells were incubated at 37 °C for 20 minutes in an HBSS solution containing the following dyes at 1 µM concentrations: poRhoVR, Rhodamine 123 and Hoechst 33342. Images were taken using W-Plan-Apo 63x/1.0 objective (Zeiss) on an upright epifluorescence microscope. poRhoVR was imaged using a red LED (Neutral Density Setting, ND, 75; 30.8 mW/mm²) as the excitation source and 100 ms exposure. Emission was collected with a single emission filter (723/68 nm) after passing through a quadruple dichroic mirror (432/38, 509/22, 586/40, 654 nm LP). Rhodamine 123 was imaged using a cyan LED (ND 75, 51.5 mW/mm²) and 100 ms exposure. Emission was collected with a single emission filter (540/50 nm) after passing through a dichroic mirror (510 nm LP). Hoechst 33342 was imaged using a violet LED (ND 75, 6.7 mW/mm²) and 100 ms exposure. Emission was collected using a triple emission filter (473/22 nm, 543/19 nm, 648/98 nm) after passing through a triple dichroic mirror (475/30 nm, 540/25 nm, 642/96 nm).

**Voltage sensitivity measurements (Figure 2 i-j)**

HEK293T cells were incubated at 37 °C for 20 minutes in an HBSS solution containing 1 µM poRhoVRs. Voltage sensitivity data was acquired using a 20X water immersion objective on an upright epifluorescence microscope. Recordings were binned 4X4 and recorded at an optical sampling rate of 500 Hz. poRhoVRs were imaged with a red LED (ND 75, 30.8 mW/mm²) and
emission was collected with the QUAD filter set (see General Imaging Parameters). For electrophysiological experiments in HEK293T cells, pipettes were pulled from borosilicate glass (Sutter Instruments, BF150-86-10), with a resistance of 4–7 MΩ, and were filled with an internal solution; 125 mM potassium gluconate, 1 mM EGTA, 10 mM HEPES, 5 mM NaCl, 10 mM KCl, 2 mM ATP disodium salt, 0.3 mM GTP trisodium salt (pH 7.25, 285 mOsm). Recordings were made with an Axopatch 200B amplifier (Molecular Devices) at room temperature. The signals were digitized with a Digidata 1440A, sampled at 50 kHz and recorded with pCLAMP 10 software (Molecular Devices) on a PC. Fast capacitance was compensated in the on-cell configuration. Recordings were only pursued if series resistance in voltage clamp was less than 30 MΩ. Cells were held at -60 mV and hyper- and de- polarizing steps applied from +100 to -100 mV in 20 mV increments. Analysis of voltage sensitivity in HEK cells was performed using custom MATLAB routines in which a region of interest (ROI) was automatically selected based on changes in fluorescence intensity. The ROI is then applied as a mask to all frames in the image stack. Fluorescence intensity values were calculated at known baseline and voltage step epochs and used to calculate a percent change in baseline fluorescence (% ΔF/F) per 100 mV change in membrane potential.

Figure 3. Voltage imaging in dissociated rat hippocampal neurons with poRhoVR 14
Dissociated rat hippocampal neurons were incubated at 37 °C for 20 min in an HBSS solution containing 500 nM poRhoVR 14. Spontaneous neuronal activity was monitored using a 20X water immersion objective on an upright epifluorescence microscope. The field of view was binned 4X4 and recorded at an optical sampling rate of 500 Hz. poRhoVR 14 was imaged using a red LED (ND 50, 21.2 mW/mm²). Emission was collected with the QUAD filter set (see General Imaging Parameters).

Figure 4. and Figure S6. All-optical electrophysiology using poRhoVR 14 and ChR2
Dissociated rat hippocampal neurons previously transfected with Channelrhodopsin-2-YFP (ChR2-YFP) were incubated at 37 °C for 20 minutes in an HBSS solution containing 500 nM poRhoVR 14. ChR2 positive cells were identified by locating YFP expressing neurons using a teal LED (50% max power, 9.2 mW/mm²). YFP emission was collected via a triple emission filter (473/22 nm, 543/19 nm, 648/98 nm) after passing through a triple dichroic mirror (475/30 nm, 540/25 nm, 642/96 nm). ChR2 was activated by 5 ms pulses of cyan light (2% of max power, 1.92 mW/mm²). poRhoVR 14 was imaged with a red LED (100% of max power, 78.5 mW/mm²) and emission was collected with the QUAD filter set (see General Imaging Parameters). The field of view was binned 4X4 and recorded at an optical sampling rate of 500 Hz using a 20X water immersion objective on an upright epifluorescence microscope.

Figure 5. Simultaneous voltage and calcium imaging with poRhoVR 14
Dissociated rat hippocampal neurons previously transfected with GCaMP6s were incubated at 37 °C for 20 minutes in an HBSS solution containing 500 nM poRhoVR 14. Separately, neurons were loaded with 1 µM OGB-AM and 500 nM poRhoVR with 0.01% pluronic. A red LED (50% of max power, 49.7 mW/mm²) was the source of excitation light for poRhoVR 14 and a cyan LED was
used for GCaMP6s (10% of max power, 7.9 mW/mm²). Emission from GCaMP6s or OGB-AM and poRhoVR 14 was collected simultaneously with the QUAD filter set and a Dual-View emission splitter (see General Imaging Parameters). Extracellular field stimulation was delivered by a Grass Stimulator connected to a recording chamber containing two platinum electrodes (Warner), with triggering provided through a Digidata 1440A digitizer and pCLAMP 10 software (Molecular Devices). Action potentials were triggered by 1 ms 80 V field potentials delivered at 5 Hz. To prevent recurrent activity the HBS bath solution was supplemented with synaptic blockers 10 μM 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX; Santa Cruz Biotechnology) and 25 μM DL-2-Amino-5-phosphonopentanoic acid (APV; Sigma-Aldrich).

Figure 6. and Figure S9. Simultaneous mapping of electrical and Ca2+activity using poRhoVR, GCaMP6s and multi-electrode 370arrays (MEA) in ex vivo retinas from rd1 mice

Retinas were dissected from 3-month-old heterozygous rd1-GCaMP6f mouse in artificial cerebral spinal fluid (ACSF). ACSF was prepared with the following: NaCl (119 mM), KCl (2.5 mM), MgCl₂ (1.3 mM), CaCl₂ (2.5), NaHCO₃ (26.2 mM), D-glucose (20 mM) and KH₂PO₄ (1 mM). GCaMP6f was expressed under a synapsin promoter. Each retina was cut into four pieces and placed into an ACSF solution of 5 μM poRhoVR 14. The solution was bubbled continuously for approximately 2 hours at room temperature with carbogen (95% O₂ and 5% CO₂). A piece of retina was placed on to an MEA chip (Multichannel Systems 60 pin ThinMEA chip, 200 µm interelectrode distance, 30 µm electrode diameter, 180 µm thickness) with the RGC layer in direct contact with the electrodes. The electrode pins on the MEA chip are made of titanium nitride (TiN) and the chip itself is made of indium tin oxide (ITO). Electrical data from the MEA was recorded on a Multichannel Systems USB-64 system at a sampling rate of 20kHz. The retina was imaged on an inverted epifluorescence microscope equipped with an OracFlash4.0 sCMOS camera (Hamamatsu) via a 20X air objective. The field of view was binned 4X4 and recorded at an optical sampling rate of 500 Hz. A red LED (20% of max power, 8.85 mW/mm²) was used as the excitation source for poRhoVR 14 and a cyan LED (20% of max power, 8.77 mW/mm²) was used for GCaMP6f. Emission from GCaMP6f and poRhoVR 14 was collected simultaneously with the QUAD filter set alongside a Dual-View emission splitter (Optical Insights). The Dual-View contained a 585dcxr dichroic and two emission filters (520/28 nm and 723/68 nm) which separated the GCaMP6f and poRhoVR 14 fluorescence signals. After a few initial recordings the ACSF solution was replaced by a solution of synaptic blockers. The solution of synaptic blockers was prepared in ACSF with the following: DNQX (40 μM), D-AP5 (30 μM), Curare (50 μM), Strychnine (10 μM), TPMPA (50 μM), Gabazine (10 μM) and L-AP4 (10 μM). Recordings of pharmacologically isolated ganglion cells were taken approximately 10-15 minutes after blocker treatment.

Figure S2. Quantification of poRhoVR staining in HEK cells.

HEK 293T cells were incubated in a 2 µM HBSS solution of one of poRhoVRs 12-15 for 20 min at 37 °C. A mixture of cis and trans poRhoVR 13 isomers was used. Images were taken using 20x water immersion objective on an upright epifluorescence microscope. poRhoVRs were imaged using a red LED (ND 75, 30.8 mW/mm²) as the excitation source and 50 ms exposure. Emission was collected with the QUAD filter set (see General Imaging Parameters). The median fluorescence of cell ROIs were compared across all four dyes.

Photophysical Characterizations
Absorbance and emission spectra were collected using a Shimadzu 2501 Spectrophotometer (Shimadzu) and a Quantamaster Master 4 L-format scanning spectrofluorometer (Photon Technologies International). The fluorometer excitation source is an LPS-220B 75-W xenon lamp. The fluorometer is equipped with a power supply, A-1010B lamp housing with integrated igniter, switchable 814 photon-counting/analog photomultiplier detection unit, and MD5020 motor driver. Samples were measured in 1-cm path length quartz cuvettes (Starna Cells).

Relative quantum yields ($\phi_F$) were measured via comparison to Cy5.5 ($\phi_{\text{standard}} = 0.23$ in PBS). DMSO stocks of fluorophores 8-9 and poRhoVRs 12-15 were diluted in PBS with 0.1% sodium dodecyl sulfate (SDS) until the max absorbance was less than 0.1 absorbance units. Serial dilutions were made from the initial dilution into PBS with 0.1% SDS. The absorbance at 625 nm versus the total area under the fluorescence curve from 635-855 nm were plotted. The slope of the resulting linear plot was calculated for each compound and for the standard. The slope was put into the equation below where; $x$ represents the compound being analyzed, $st$ represents the standard, $\phi_x$ is the $\phi_F$ for compound x and $\eta$ is the refractive index of the solvent in which the measurements were made. The absorbance and fluorescence measurements for fluorophores 8 and 9 were made in PBS and in PBS with 0.1% SDS for poRhoVRs 12-15. For both solutions the refractive index was approximated to the refractive index of water ($\eta = 1.33$).

$$\phi_x = \phi_{st} \left( \frac{\text{Slope}_x}{\text{Slope}_{st}} \right) \left( \frac{\eta_x^2}{\eta_{st}^2} \right)$$

**General Synthesis and Characterization Information**

Chemical reagents and solvents were purchased from commercial sources and used without further purification. Flash column chromatography was performed using Silicycle Silica Flash F60 (230–400 Mesh). Preparative Thin Layer Chromatography (PTLC) purification was done using glass plates coated with a layer of silica gel (Silicycle, F254, 1000 µm). Preparative High Performance Liquid Chromatography (Prep-HPLC) was done using a Waters Acquity Autopurification system equipped with a Waters XBridge BEH 5 µm C18 column (19 mm I.D. x 250 mm) with a flow rate of 30.0 mL/min, made available by the Catalysis Facility of Lawrence Berkeley National Laboratory (Berkeley, CA). The mobile phases were MiliQ (MQ-H$_2$O) with 0.05% formic acid (FA) and HPLC grade acetonitrile (MeCN) with 0.05% FA. Absorbance was monitored at 350 nm over 200 min with a gradient of 10-100% of MeCN with 0.05% FA. NMR spectra were collected on one of the following instruments; Bruker AVB-400 MHz, AVQ-400 MHz, Bruker AV-600 MHz. Chemical shifts are reported in parts per million (ppm) and couplings constants are reported in Hertz (Hz). Characterization of purity and identity was also done via High Performance Liquid Chromatography (HPLC) and low resolution ESI mass spectrometry on an Agilent Infinity 1200 analytical instrument coupled to an Advion CMS-L ESI mass spectrometer. The column used for the analytical HPLC was Phenomenex Luna 5 µm C18(2) (4.6 mm I.D. x 75 mm) with a flow rate of 1.0 mL/min. The mobile phases were MQ-H$_2$O with 0.05% formic acid (FA) and HPLC grade MeCN with 0.05% FA. Absorbance was monitored at 254, 280, 350 and 690 nm over 10 min with a gradient of 10-100% of MeCN with 0.05% FA.

**Crystallization of 8- and 9- cis**
Crystals were grown through vapor diffusion. The compound was dissolved in a mixture of 90% 1,2-dichloroethane, 5% ethanol, 5% methanol by volume. The resulting solution was filtered with a 0.45 µm PTFE filter into a 60 mm test tube. The tube was placed into a 20 mL vial containing 7 mL of hexanes and capped with lid (foil inner lining). The vial was kept at room temperature away from light.

Single-crystal X-ray diffraction experiments were performed at the UC Berkeley CHEXRAY crystallographic facility. Measurements of all compounds were performed on a Bruker Quazar SMART APEX-II using Mo Kα radiation (λ = 0.71073 Å). Crystals were kept at 100(2) K throughout collection. Data collection was performed with Bruker APEX2 software (v. 2014.11). Data refinement and reduction were performed with Bruker SAINT (V8.34A). All structures were solved with SHELXT.53. Structures were refined with SHELXL-2016. Molecular graphics were computed with Mercury 4.0. All non-hydrogen atoms were refined anisotropically, and hydrogen atoms were included at the geometrically calculated positions and refined using a riding model.

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NMR Abbreviations

s = singlet

d = doublet

dd = doublet of doublets

t = triplet

dt = doublet of triplets

td = triplet of doublets

q = quartet

qnt = quintet

dqnt = doublet of quintets

m = multiplet

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Synthesis of methyldiphenylphosphine oxide, 2:

Added 50 mL of H₂O to 1 (5.00 g, 14.0 mmol). Refluxed the solution for 30 minutes. Made a separate solution of NaOH (2.80 g, 70.0 mmol) in 25 mL of H₂O. The solution of NaOH was added to the solution of 1 and the reaction mixture became cloudy and opaque. The mixture was refluxed for an additional 2 hours. Once the mixture cooled to room temperature it was extracted with CHCl₃ (3X). The organic layer was washed with H₂O (3X), collected and dried with anhydrous Na₂SO₄. The solvent was removed by rotary evaporation. Collected 3.02 g of a white solid (quantitative yield). ¹H NMR (400 MHz, CDCl₃) δ7.754-7.700 (m, 4 H); δ7.528-7.445 (m, 6 H); δ2.041-2.008 (d, J=13.2, 3 H).

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Synthesis of methylbis(3-nitrophynyl)phosphine oxide, 3:

Dissolved 2 (1.00 g, 4.63 mmol) in 5.2 mL of concentrated H₂SO₄. The solution was cooled to 0 °C. A separate solution of concentrated H₂SO₄ (1.8 mL) and HNO₃ (1.0 mL) was made while on
ice. The acid mixture was added dropwise to the solution of 2. After addition was complete the reaction mixture changed from clear to yellow. The reaction was stirred at 0 °C for two hours and then for an additional 3 hours at room temperature. The mixture was poured over ice and extracted with CHCl₃. The organic layer was collected and washed with a saturated NaHCO₃ solution (3X) and brine (1X). The organic layer was dried over anhydrous Na₂SO₄ and the solvent removed by rotary evaporation. Collected 1.40 g of a pale yellow solid (quantitative yield). ¹H NMR (400 MHz, CDCl₃) δ8.577-8.538 (dt, J=1.8 Hz, J=12.2 Hz, 2 H); δ8.441-8.414 (dqnt, J=1.1 Hz, J=8.2 Hz, 2H); δ8.154-8.107 (m, 2 H); δ7.777-7.731 (td, J=2.72 Hz, J=7.9 Hz, 2 H); δ2.221-2.188 (d, J=13.4 Hz, 3 H).

Synthesis of bis(3-aminophenyl)(methyl)phosphine oxide, 4:

Added 3 (0.25 g, 0.82 mmol) and 5% Pd/C (0.025 g, 10% wt) to a Schlenk flask. Evacuated the flask and backfilled using a balloon filled with H₂. Added 10 mL of MeOH and stirred at room temperature for 12 hours. The reaction mixture was filtered through a pad of Celite. The pad was washed with acetone and the filtrate collected. The solvent was removed by rotary evaporation. Collected 0.20 g of crude product. ¹H NMR (400 MHz, MeOD) δ7.245-7.197 (td, J=3.8 Hz, J=7.7 Hz, 2 H); δ7.016-6.936 (m, 4H); δ6.876-6.856 (dqnt, J=1.1 Hz, J=8.1 Hz, 2 H); δ2.221-2.188 (d, J=13.4 Hz, 3 H).

Synthesis of bis(3-(diethylamino)phenyl)(methyl)phosphine oxide, 5:

To 4 (5.83 g, 23.7 mmol) added 10 mL of dry DMF, EtI (10.0 mL, 124.4 mmol) and K₂CO₃ (16.4 g, 118.7 mmol). Refluxed for 12 hours. Removed DMF from reaction mixture by rotary evaporation. The remaining solid was redissolved in H₂O and extracted with DCM (3X). The organics layer was dried over Na₂SO₄ and the solvent removed by rotary evaporation. The resulting residue was purified via flash column chromatography using silica as the stationary phase and 3% MeOH in DCM as the mobile phase. Collected 7.6 g of a golden oil (89% yield). ¹H NMR (400 MHz, CDCl₃) δ7.260-7.217 (m, 2 H); δ 7.108-7.073 (dm, J=13.9 Hz, 2 H); δ6.880-6.833 (m, 2 H); δ6.772-6.746 (dm, J=8.3 Hz, 2H); δ3.378-3.326 (q, J=7.1Hz, 8 H); δ1.973-1.940 (d, J=13.1 Hz, 3 H); δ1.143-1.108 (t, J=7.0 Hz, 12 H).
Synthesis of \( m \)-bromo tetraethylphosphourous rhodamine (mBrTEPR), 8:

Dissolved 5 (0.100 g, 0.280 mmol), 6 (0.067 g, 0.25 mmol) and urea (0.030 g, 0.50 mmol) in 2 mL of glacial acetic acid. Stirred at 110 °C for 24 hours. The reaction mixture changed from clear yellow to a dark blue green. Once the mixture had cooled to room temperature the acetic acid was removed by rotary evaporation. The blue green residue was redissolved in saturated NaHCO\(_3\) and extracted with 2:1 DCM:IPA (3X). The organic layer was collected and dried over Na\(_2\)SO\(_4\) and the solvent removed by rotary evaporation. The product was obtained by purifying first with a silica column using a gradient from 2-20% MeOH in DCM, followed by a basic alumina column using a gradient from 0-2% MeOH in DCM. The first blue green band to elute was collected which corresponds to the cis isomer of 8. The second blue green band to elute was also collected and contained the trans isomer of 8 but required further purification. The trans isomer was obtained using reverse phase HPLC using a gradient from 0-100% MeCN in H\(_2\)O with 0.05% formic acid. Collected 0.015 g of the major cis isomer as a black blue solid (10 % yield) and 1.6 mg of the minor trans isomer (1.1% yield). 8 (cis) \(^1\)H NMR (400 MHz, MeOD) \( \delta \) 8.041-8.020 (d, \( J=8.4\ \text{Hz}, 1\ \text{H} \)); \( \delta \) 7.868-7.842 (dd \( J=2.0\ \text{Hz}, J=8.4\ \text{Hz}, 1\ \text{H} \)); \( \delta \) 7.686-7.639 (dd \( J=2.8\ \text{Hz}, J=16.0\ \text{Hz}, 2\ \text{H} \)); \( \delta \) 7.478-7.473 (d \( J=2.0\ \text{Hz}, 1\ \text{H} \)); \( \delta \) 7.111-7.073 (m, 2 H); \( \delta \) 66.930-6.889 (dd \( J=2.8\ \text{Hz}, J=9.6\ \text{Hz}, 2\ \text{H} \)); \( \delta \) 53.838-3.722 (m, 8 H); \( \delta \) 52.018-1.983 (d, \( J=14.0\ \text{Hz}, 3\ \text{H} \)); \( \delta \) 1.355-1.319 (t, \( J=7.2\ \text{Hz}, 12\ \text{H} \)). \(^{31}\)P NMR (400 MHz, MeOD) \( \delta \) 20.375. 8 (trans) \(^1\)H NMR (400 MHz, MeOD) \( \delta \) 8.057-8.036 (d, \( J=8.4\ \text{Hz}, 1\ \text{H} \)); \( \delta \) 7.863-7.837 (dd \( J=2.0\ \text{Hz}, J=8.4\ \text{Hz}, 1\ \text{H} \)); \( \delta \) 7.677-7.630 (dd \( J=2.8\ \text{Hz}, J=16.0\ \text{Hz}, 2\ \text{H} \)); \( \delta \) 7.545-7.540 (d \( J=2.0\ \text{Hz}, 1\ \text{H} \)); \( \delta \) 7.127-7.088 (m, 2 H); \( \delta \) 66.915-6.884 (dd \( J=2.8\ \text{Hz}, J=9.6\ \text{Hz}, 2\ \text{H} \)); \( \delta \) 53.845-3.641 (m, 8 H); \( \delta \) 51.963-1.929 (d, \( J=13.6\ \text{Hz}, 3\ \text{H} \)); \( \delta \) 1.354-1.318 (t, \( J=7.2\ \text{Hz}, 12\ \text{H} \)). \(^{31}\)P NMR (400 MHz, MeOD) \( \delta \) 18.389. ESI MS, calculated for [M+], \( C_{28}H_{33}BrN_2O_4PS^+ \), 603.11; found: 603.1. HR-ESI-MS, calculated for [M+], \( C_{28}H_{33}BrN_2O_4PS^+ \), 603.1075, found 603.1075.
Synthesis of p-bromo tetraethylphosphourous rhodamine (pBrTEPR), 9:

Dissolved 5 (0.100 g, 0.280 mmol), 7 (0.067 g, 0.25 mmol) and urea (0.030 g, 0.50 mmol) in 2 mL of glacial acetic acid. Stirred at 110 °C for 24 hours. The reaction mixture changed from clear yellow to a dark blue green. Once the mixture had cooled to room temperature the acetic acid was removed by rotary evaporation. The blue green residue was redissolved in saturated NaHCO₃ and extracted with 2:1 DCM:IPA (3X). The organic layer was collected and dried over Na₂SO₄ and the solvent removed by rotary evaporation. The product was obtained by purifying first with a silica column using a gradient from 2-20% MeOH in DCM, followed by a basic alumina column using a gradient from 0-2% MeOH in DCM. The first blue green band to elute was collected which corresponds to the trans isomer of 9. Collected 0.007 g of the cis isomer as a black blue solid (5 % yield). The second blue green band to elute contained the minor trans isomer with an impurity that had a mass corresponding to the dibrominated product. The trans isomer further purified via reverse phase HPLC using a gradient from 0-100% MeCN in H₂O with 0.05% formic acid. Collected 0.7 mg of the minor trans isomer (0.46 % yield). 9 (cis) ¹H NMR (400 MHz, MeOD) δ8.268-8.264 (d, J=2.0 Hz, 1 H); δ7.825-7.805 (dd, J=2.0 Hz, J=8.1 Hz, 1 H); δ7.678-7.640 (dd, J=2.7 Hz, J=15.9 Hz, 2 H); δ7.194-7.177 (d, J=8.1 Hz, 1 H); δ7.102-7.072 (m, 2 H); δ6.914-6.890 (dd, J=2.5 Hz, J=9.6 Hz, 2 H); δ5.379-3.725 (m, 8 H); δ2.015-1.987 (d, J=14.0 Hz, 3 H); δ 1.345-1.317 (t, J=6.9 Hz, 12 H). ³¹P NMR (400 MHz, MeOD) δ20.325. 9 (trans) ¹H NMR (400 MHz, MeOD) δ8.286-8.281 (d, J=2.0 Hz, 1 H); δ7.800-7.775 (dd, J=2.0 Hz, J=8.2Hz, 1 H); δ7.673-7.627 (dd, J=2.6 Hz, J=16 Hz, 2 H); δ7.232-7.212 (d, J=8.2Hz, 1 H); δ7.124-7.085 (m, 2 H); δ 6.905-6.875 (dd, J=2.6 Hz, J=9.6 Hz); δ3.841-3.687 (m, 8 H); δ 1.948-1.914 (d, J=13.5 Hz); δ1.349-1.313 (t, J=7.0 Hz, 12 H). ³¹P NMR (400 MHz, MeOD) δ18.217. ESI MS, calculated for [M+][⁺], C₂₇H₂₅BrN₂O₄PS⁺, 603.11; found: 603.1. HR-ESI-MS, calculated for [M+][⁺], C₂₇H₂₅BrN₂O₄PS⁺, 603.1077; found 603.1079, calculated for [M-H+Na][⁺], C₂₈H₃₂BrN₂O₄PSNa⁺, 625.0896; found 625.0899.
Synthesis of 12:

In an oven-dried flask added 8 (25 mg, 0.042 mmol), 10 (10 mg, 0.042 mmol), Pd(OAc)$_2$ (2.4 mg, 0.011 mmol) and P(o-tol)$_3$ (6.4 mg, 0.029 mmol). Evacuated and back-filled the flask with N$_2$ (3X). Dissolved the solids in 0.4 mL of dry DMSO and added dry NEt$_3$ (0.12 mL). Stirred at 75 °C for 18 hours. Once the reaction mixture cooled it was diluted with DCM and washed with H$_2$O (3X). The organic layer was washed with brine (1X) and dried over Na$_2$SO$_4$. The solvent was removed by rotary evaporation and the resulting residue was purified via prep TLC using 10% MeOH in DCM as the mobile phase. Further purified via reverse phase HPLC using a gradient from 0-100% MeCN in H$_2$O with 0.05% formic acid. Collected 1.1 mg of product (3.0 % yield).

$^1$H NMR (400 MHz, MeOD) $\delta$8.110-8.092 (d, J=8.3 Hz, 1 H); $\delta$7.874-7.851 (dd, J=1.4 Hz, J=8.3 Hz, 1 H); $\delta$7.682-7.651 (dd, J=2.7 Hz, J=15.8 Hz, 2 H); $\delta$7.543-7.529 (d, J=8.3 Hz, 2 H); $\delta$7.489-7.475 (d, J=8.3 Hz, 2 H); $\delta$7.422-7.408 (m, 3 H); $\delta$7.347-7.319 (d, J=16.4 Hz, 1 H); $\delta$7.243-7.189 (m, 3 H); $\delta$7.116-7.089 (d, J=16.3 Hz, 1 H); $\delta$5.966 (s, 3.6 H); $\delta$2.031-2.008 (d, J=14 Hz, 3 H); $\delta$1.353-1.336 (t, J=6.9 Hz, 10 H). ESI MS, calculated for [M+H]$^+$, C$_{46}$H$_{51}$N$_3$O$_4$PS$^+$, 386.67; found: 386.6. HR-ESI-MS, calculated for [M+], C$_{46}$H$_{52}$N$_3$O$_4$PS, 772.3332; found 772.3321, calculated for [M-H+Na]$^+$, C$_{46}$H$_{50}$N$_3$O$_4$PSNa$^+$, 794.3152; found 794.3149,
Synthesis of 13:

In an oven-dried flask added 8 (14.3 mg, 0.0237 mmol), 11 (7.3 mg, 0.024 mmol), Pd(OAc)$_2$ (2.7 mg, 0.012 mmol) and P(o-tol)$_3$ (7.2 mg, 0.024 mmol). Evacuated and back-filled the flask with N$_2$ (3X). Dissolved the solids in 1 mL of dry DMF and added NEt$_3$ (0.13 mL). Stirred at 75 °C for 2 hours before adding another 2.7 mg of Pd(OAc)$_2$. After the second addition of catalyst allowed the reaction to stir for an additional 12 hours. The product was obtained by purifying the reaction mixture via prep TLC using 10% MeOH in DCM as the mobile phase. Collected a green solid that was a mixture of isomers (9.8 mg, 50% yield). To separate the two isomers further purification was required. The solvent was removed from the reaction mixture and the remaining residue was first purified on an alumina column using 0-5% MeOH in DCM as the mobile phase. Two green solids were collected and are further purified via reverse phase HPLC with 0-100% MeCN in H$_2$O with 0.05% formic acid. Collected 1.8 mg of the major cis product (9.0% yield) and 0.2 mg of the minor trans product (1.0% yield).

$^{1}$H NMR (400 MHz, MeOD) δ8.054-8.033 (d, J=8.3 Hz, 1 H); δ7.766-7.740 (dd, J=1.76 Hz, J=8.3 Hz, 1 H); δ7.652-7.605 (dd, J=2.7 Hz, J=15.9 Hz, 2 H); δ7.545-7.344 (m, 7 H); δ7.266-7.226 (dd, J=16.3 Hz, 1 H); δ7.163-7.100 (m, 3 H); δ6.902-6.861 (d, J=16.4 Hz, 1 H); δ6.822-6.791 (dd, J=2.7 Hz, J=9.7 Hz, 2 H); δ6.345-6.317 (dd, J=2.4 Hz, J=8.8 Hz, 1 H); δ6.253-6.247 (d, J=2.4 Hz, 1 H); δ3.873 (s, 3 H); δ3.757-3.722 (m, 8 H); δ3.444-3.392 (q, J=7.0 Hz, 4 H); δ3.843-3.691 (m, 8 H); δ3.446-3.394 (q, J=7.0 Hz, 4 H); δ2.006-1.972 (d, J=13.5 Hz, 3 H); δ1.354-1.319 (t, J=6.9 Hz, 12 H); δ1.205-1.170 (t, J=7.0 Hz, 6 H).

$^{31}$P NMR (400 MHz, MeOD) δ20.204.

ESI MS, calculated for [M+Na]$^+$, C$_{49}$H$_{57}$N$_3$O$_5$PS$^+$, 852.3570; found 852.3570.
Synthesis of 14:

In an oven-dried flask added 9 (5.8 mg, 0.0096 mmol), 10 (2.5 mg, 0.0096 mmol), Pd(OAc)$_2$ (1.1 mg, 0.0048 mmol) and P(o-tol)$_3$ (2.9 mg, 0.0038 mmol). Evacuated and back-filled the flask with N$_2$ (3X). Dissolved the solids in 1 mL of dry DMF and added NEt$_3$ (0.05 mL). Stirred at 75 °C for 2 hours. The product was obtained by purifying the reaction mixture via prep TLC using 10% MeOH in DCM as the mobile phase. Collected 6.7 mg of a green solid (90% yield). $^1$H NMR (600 MHz, MeOD) δ8.550 (s, 1 H, formate); δ8.313-8.311 (d, J=0.8 Hz, 1 H); δ7.833-7.818 (dd, J=0.9 Hz, J=8.8 Hz, 1 H); δ 7.672-7.642 (dd, J=2.5 Hz, J=15.8 Hz, 2 H); δ7.607-7.593 (d, J=8.2 Hz, 2 H); δ 7.537-7.524 (d, J=8.2 Hz, 2 H); δ7.440-7.426 (d, J=8.7 Hz, 2 H); δ7.409-7.381 (d, J=16.4 Hz, 1 H); δ 7.346-7.319 (d, J=16.4 Hz, 1 H); δ 7.223-7.173 (m, 3 H); δ7.147-7.120 (d, J=16.3 Hz, 1 H); δ6.976-6.949 (d, J=16.3 Hz, 1 H); δ6.906-6.885 (dd, J=2.6 Hz, J=9.7 Hz, 2 H); δ6.776-6.762 (d, J=8.7 Hz, 2 H); δ3.813-3.716 (m, 8 H); δ2.030-2.007 (d, J=13.9 Hz, 3 H); δ1.343-1.321 (t, J=6.5 Hz, 12 H). $^{31}$P NMR (400 MHz, MeOD) δ20.359. ESI MS, calculated for [M$^+$]$^+$, C$_{46}$H$_{51}$N$_3$O$_4$PS$^+$, 772.33; found 772.3; [M$^+$]$^+$, C$_{46}$H$_{52}$N$_3$O$_4$PS$^+$, 386.67; found: 386.7. HR-ESI-MS, calculated for [M$^+$]$^+$, C$_{46}$H$_{51}$N$_3$O$_4$PS$^+$, 772.3332; found 772.3332; calculated for [M$^+$]$^+$, C$_{46}$H$_{52}$N$_3$O$_4$PS$^+$, 386.6703; found 386.6707, calculated for [M$^+$+Na]$^+$, C$_{46}$H$_{51}$N$_3$O$_4$PSNa$^+$, 397.6612; found 397.6617.
Synthesis of 15:

In an oven-dried flask added 9 (50 mg, 0.083 mmol), 11 (26 mg, 0.083 mmol), Pd(OAc)$_2$ (4.7 mg, 0.021 mmol) and P(o-tol)$_3$ (12.8 mg, 0.042 mmol). Evacuated and back-filled the flask with N$_2$ (3X). Dissolved the solids in 0.5 mL of dry DMF and added dry NEt$_3$ (0.02 mL). Stirred at 75 °C for 12 hours. The product was obtained by purifying the reaction mixture via prep TLC using 10% MeOH in DCM as the mobile phase, further purified via reverse phase HPLC using a gradient from 0-100% MeCN in H$_2$O with 0.05% formic acid. Collected 4.5 mg of a green solid (6.5% yield).

$^1$H NMR (600 MHz, MeOD) δ8.305 (s, 1 H); δ7.809-7.796 (d, J=7.92 Hz, 1 H); δ7.665-7.635 (dd, J=2.34 Hz, J=15.78 Hz, 2 H); δ7.573-7.560 (d, J=8.1 Hz, 2 H); δ7.485-7.472 (d, J=8.1 Hz, 2 H); δ7.448-7.406 (m, 2 H); δ7.387-7.360 (d, J=16.3 Hz, 1 H); δ7.318-7.291 (d, J=16.3 Hz, 1 H); δ7.203-7.167 (m, 3 H); δ6.948-6.921 (d, J=16.4 Hz, 1 H); δ6.931-6.870 (dd, J=2.5 Hz, J=12.2 Hz, 2 H); δ6.351-6.234 (dd, J=1.3 Hz, J=8.7 Hz, 1 H); δ6.266-6.264 (d, J=1.5 Hz, 1 H); δ3.887 (s, 3 H); δ3.442-3.407 (q, J=7.0 Hz, 4 H); δ3.887 (s, 3 H); δ2.026-2.002 (d, J=14.0 Hz, 3 H); δ1.339-1.316 (t, J=6.8 Hz, 12 H); δ1.206-1.83 (t, J=7.0 Hz, 6 H).  $^{31}$P NMR (400 MHz, MeOD) δ20.322.

ESI MS, calculated for [M+H]$^+$, C$_{49}$H$_{56}$N$_3$O$_5$PS$^+$, 415.69; found: 415.7. HR-ESI-MS, calculated for [M+]$^+$, C$_{49}$H$_{57}$N$_3$O$_5$PS$^+$, 830.3751; found 830.3729, calculated for [M+Na]$^+$, C$_{49}$H$_{56}$N$_3$O$_5$PSNa$^+$, 852.3571; found 852.3570.

Synthesis of 6, 7, 10 & 11:

Synthesis of these compounds can be found in Kulkarni, R.U.; Yin, H.; Pourmandi, N.; James, F.; Adil, M.M.; Schaffer, D.V.; Wang, Y.; Miller, E.W.; ACS Chem. Biol. 12, 407–413 (2017)

References

Supplemental Spectra

Spectrum S1. $^1H$ NMR methyldiphenylphosphine oxide, 2
Spectrum S2. $^1$H NMR methylbis(3-nitrophenyl)phosphine oxide, 3
Spectrum S3. $^1$H NMR bis(3-aminophenyl)(methyl)phosphine oxide, 4
Spectrum S4. $^1$H NMR bis(3-(diethylamino)phenyl)(methyl)phosphine oxide, 5
Spectrum S5. $^1$H NMR m-bromosulfo-phosphine oxide rhodamine (cis), 8

Spectrum S6. $^{31}$P NMR m-bromosulfo-phosphine oxide rhodamine (cis), 8
Spectrum S7. LC/MS traces m-bromosulfo-phosphine oxide rhodamine (cis), 8
Spectrum S8. $^1$H NMR m-bromosulfo-phosphine oxide rhodamine (trans), 8

Spectrum S9. $^{31}$P NMR m-bromosulfo-phosphine oxide rhodamine (trans), 8
Spectrum S10. LC/MS traces m-bromosulfo-phosphine oxide rhodamine (trans), 8
Spectrum S11. $^1$H NMR p-bromosulfo-phosphine oxide rhodamine (cis), 9

Spectrum S12. $^{31}$P NMR p-bromosulfo-phosphine oxide rhodamine (cis), 9
Spectrum S13. LC/MS traces p-bromosulfo-phosphine oxide rhodamine (cis), 9
Spectrum S14. $^1$H NMR p-bromosulfo-phosphine oxide rhodamine (trans), 9

Spectrum S15. $^{31}$P NMR p-bromosulfo-phosphine oxide rhodamine (trans), 9
Spectrum S16. LC/MS traces p-bromosulfo-phosphine oxide rhodamine (trans), 9
Spectrum RT 4.76 - 5.17 (F3 scans)

intensity

mass (m/z)
Spectrum S17. $^1$H NMR poRhoVR (cis), 12

Spectrum S18. $^{31}$P NMR poRhoVR (cis), 12
Spectrum S19. LC/MS traces poRhoVR (cis), 12
Spectrum S20. $^1$H NMR poRhoVR (cis), 13

Spectrum S21. $^{31}$P NMR poRhoVR (cis), 13
Spectrum S25. LC/MS traces poRhoVR (trans), 13
Spectrum S26. $^1$H NMR porHovR (cis), 14

Spectrum S27. $^{31}$P NMR porHovR (cis), 14
Spectrum S28. LC/MS traces poRhoVR (cis), 14
Spectrum S29. $^1$H NMR poRhoVR (cis), 15

Spectrum S30. $^{31}$P NMR poRhoVR (cis), 15

m/z (+1) = 829.4 g/mol
Spectrum S31. LC/MS traces poRhoVR (cis).