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Phospha-fluorescein: A Red-Emissive Fluorescein Analogue with High Photobleaching Resistance†

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Phospha-fluorescein (POF), a phosphine oxide-containing analogue of fluorescein, was synthesized and its photophysical properties were examined. Compared with fluorescein and sila-fluorescein, POF displayed significantly red-shifted absorption and fluorescence as well as superior photobleaching resistance, while retaining the pH-responsive characteristics of fluorescein dyes.

Small-molecule fluorescent probes have garnered increasing attention as useful tools for the non-invasive visualization of living tissue and biological events that cannot be visualized by fluorescent proteins.[1] Among various small fluorescent molecules, fluorescein is one of the most commonly used fluorophores, due to its high solubility in water, large molar absorption coefficient, and high fluorescence quantum yield. The fluorescein skeleton has been widely used as a core for various fluorescent probes that are sensitive to pH changes, metal ions, enzyme activity, protein expression, or reactive oxygen and nitrogen species.[2] However, fluorescein still suffers from several drawbacks, e.g. an emission wavelength in the green region, which is subject to severe autofluorescence interference, and substantial photobleaching under photoirradiation conditions.

To overcome these shortcomings and impart fluorescein with superior functionality, various structural modifications have been implemented in the past. One promising approach is the incorporation of main group elements into the π-skeleton.[3] For instance, the replacement of the endocyclic oxygen atom in the xanthene skeleton with heavier elements, such as Si and Ge (group 14),[4,5] or S, Se, and Te (group 16),[6,7] successfully furnished various useful small molecule-based probes with characteristic properties, including e.g. a red-shifted emission compared to the parent xanthene dyes.[8]

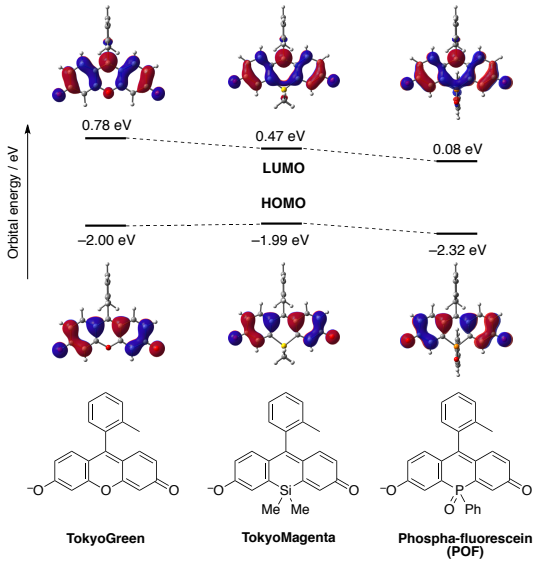

Fig. 1 Energy diagrams and Kohn–Sham HOMOs and LUMOs for the deprotonated forms of *o*-tolyl-substituted fluorescein (TokyoGreen) and the relevant heteroatom-embedded derivatives with silicon (TokyoMagenta) and phosphine oxide (phospha-fluorescein, POF) calculated at the B3LYP/6-31+G(d) level of theory.

For the electronic modification of π-conjugated skeletons, phosphorus (group 15) is also particularly useful,[9] as the orbital interaction between the π-skeleton and the phosphorus moiety significantly perturbs the electronic structure. The oxidation of the phosphorus atom to the corresponding phosphine oxide or sulfide further enhances the electron-accepting ability, as well as the chemical stability of the π-skeleton. Taking advantage of these features, various phosphine oxide-based materials have been developed for applications in organic electronic materials and fluorescent probes. We have also contributed to this research area by developing novel π-electron systems, containing phosphine oxide,[10–12] sulfide,[10c] or phosphonium moieties.[13] Throughout these studies, we have demonstrated that these structural modifications result in a bathochromic shift of the maximum wavelengths for both absorption and fluorescence. We

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†Electronic Supplementary Information (ESI) available: Experimental details, photophysical data, the results of theoretical calculations, and cell imaging experiments. See DOI: 10.1039/x0xx00000x

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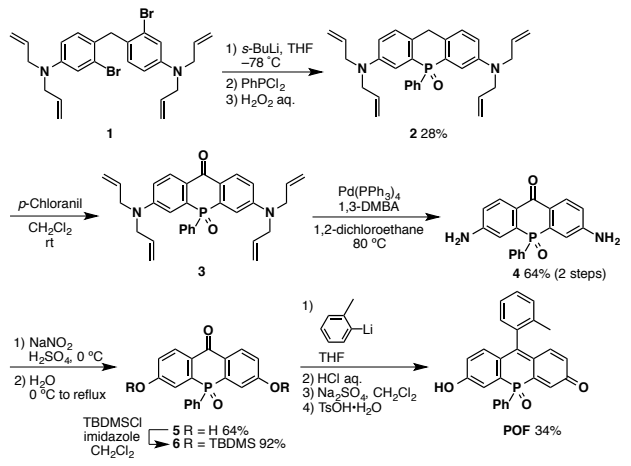

Scheme 1 Synthesis of POF.

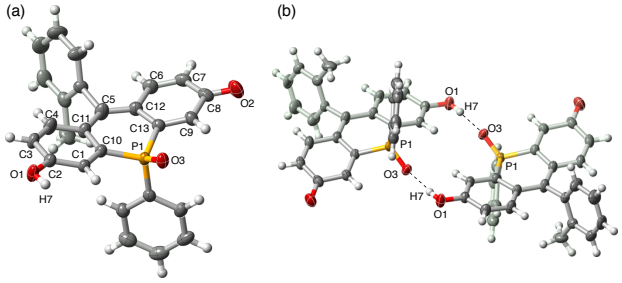

Fig. 2 (a) Molecular structure of POF and (b) bimolecular hydrogen bonding in the solid state (atomic displacement parameters set at 50% probability; only selected atoms are labelled; colour scheme: grey = carbon; white = hydrogen; red = oxygen; orange = phosphorus). Selected bond lengths (Å): P1–C10 = 1.785(2), P1–C13 = 1.7960(18), P1–O3 = 1.4890(16), C1–C2 = 1.400(2), C2–C3 = 1.392(2), C3–C4 = 1.377(2), C4–C11 = 1.409(2), C10–C11 = 1.411(2), C5–C11 = 1.471(2), C5–C12 = 1.377(3), C6–C7 = 1.339(3), C7–C8 = 1.448(3), C8–C9 = 1.469(2), C9–C13 = 1.352(2), C12–C13 = 1.454(2), C2–O1 = 1.343(2), C8–O2 = 1.238(2).

envisioned that the substitution of the endocyclic oxygen atom in the fluorescein skeleton with a phosphine oxide moiety could allow access to a new class of fluorescein dyes with an emission spectrum that should reach the far-red/near-infrared region. Based on quantum chemical calculations at the B3LYP/6-31+G(d) level of theory (Fig. 1), the replacement of the endocyclic oxygen atom in a fluorescein derivative (TokyoGreen) with a phosphine oxide moiety should result in a significant decrease of the LUMO level, due to the effective σ^*-π^* conjugation, as well as in a narrower HOMO–LUMO gap relative to the corresponding silicon-containing analogue (TokyoMagenta) on account of the strong electron-withdrawing effect of the phosphine oxide moiety. We herein report the synthesis and photophysical properties of phospha-fluorescein (POF), which is a phosphine oxide analogue of fluorescein and thus represents a new heteroatom-embedded fluorescein dye.[14]

POF was synthesized in six steps from bis(4-diallylamino-2-bromophenyl)methane **1** (Scheme 1):[4] dilithiation of **1** with *s*-BuLi, followed by successive treatment with $PhPCl_2$ and an aqueous solution of H_2O_2 produced phosphoryl-bridged diarylmethane **2** in 28% yield. Subsequently, **2** was transformed into the corresponding bis(*N*,*N*-diallylamino)-xanthone **3**. Deprotection of the amino group in **3**, followed by a Sandmeyer reaction and hydrolysis afforded phosphoryl-bridged dihydroxyxanthone **5**. Treatment of **5** with *t*-butyldimethylsilyl chloride (TBDMSCl) in the presence of imidazole afforded TBDMS-protected dihydroxyxanthone **6**. Finally, a nucleophilic addition of *o*-tolyllithium, followed by deprotection of the TBDMS groups and dehydroxylation in the presence of *p*-toluenesulfonic acid afforded POF in 34% yield. The structure of POF was unequivocally assigned on the basis of NMR and mass spectrometry, as well as single-crystal X-ray diffraction analysis.

Fig. 2 displays the molecular structure of POF, obtained from the X-ray diffraction analysis. In the crystal structure, the phosphorus-containing six-membered ring adopts an envelope-like geometry, probably due to the long endocyclic P–C bonds. The dihedral angle between the two benzene rings in the phospha-xanthene framework is 19.03°, which is small enough to allow an effective expansion of the π-conjugation. In contrast to fast tautomerism in solution, the xanthene framework of POF adopts an unsymmetrical geometry in the solid state. The C2–O1 bond (1.343(2) Å) is substantially elongated relative to the C8–O2 bond (1.238(2) Å). Also, the six-membered ring C6–C7–C8–C9–C13–C12 exhibits higher levels of bond alternation compared to the six-membered ring C1–C2–C3–C4–C11–C10. The crystal packing revealed a dimeric structure for POF, which exhibited complementary intermolecular hydrogen bonds between the phenolic hydroxy groups and the phosphine oxide moieties. The interatomic distance between the O1 and O3 atoms in the adjacent molecules is short (2.621 Å), indicating a strong hydrogen bond between the P=O and phenolic OH moieties.

The photophysical properties of POF were investigated in aqueous buffer solutions of varying pH value, containing 1% DMSO as a co-solvent (Fig. 3, Table 1, and Figs. S1–3 in the ESI). Similar to previously reported fluorescein derivatives, POF exhibited pH-dependent absorption spectra, associated with the equilibrium between the protonated and deprotonated forms of the phenolic hydroxy group. Thus, upon increasing the pH value of the solution from 3 to 11, the broad absorption band at λ_{abs} = 488 nm (ε = 15600 $M^{-1}cm^{-1}$) significantly diminished, while a new absorption band at λ_{abs} = 627 nm (ε = 51300 $M^{-1}cm^{-1}$) emerged simultaneously. An isosbestic point was observed at 532 nm (Fig. 3a and Fig. S1 in the ESI). The red-shifted absorption band should be assigned to the anionic form of POF. Based on a plot of the absorbance change at 627 nm, a pK_a value of 5.7 was determined for POF (Fig. S3a), which is lower than those of TokyoGreen (pK_a = 6.2)[15] and TokyoMagenta (pK_a = 6.8).[4] The higher acidity of the phenolic proton in POF should be correlated with the strong electron-withdrawing effect of the phosphine oxide moiety, which stabilizes the anionic form after deprotonation.

Irrespective of the pH values, POF showed an intense deep red fluorescence with a maximum at λ_{em} = 656 nm. The fluorescence spectrum at lower pH values showed two emission bands: one weak band around λ_{em} = 600 nm and one intense band at λ_{em} = 656 nm. These should be assigned to the neutral and the anionic forms, respectively. Even at the lower

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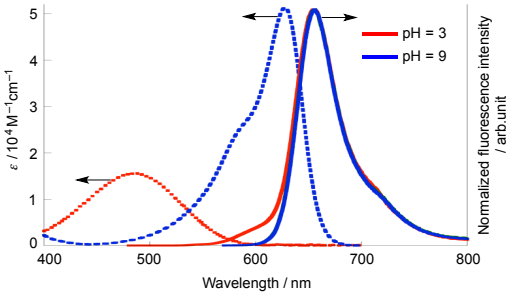

Fig. 3 UV-vis absorption (dotted lines) and fluorescence spectra (solid lines) of POF in pH-buffer solutions (pH = 3: 50 mM citric acid/sodium citrate, pH = 9: 100 mM Na_2CO_3/$NaHCO_3$), containing 1% DMSO. Fluorescence spectra were recorded with excitation wavelengths of 460 and 550 nm for pH = 3 and 9, respectively.

Table 1 Comparison of the Photophysical Properties and pK_a Values of POF and Other Relevant Fluorescein Derivatives.

Compound	Absorption		Fluorescence		pK_a
	λ_{abs} / nm	ε / 10^4 M^{-1} cm^{-1}	λ_{em} / nm	Φ_{FL}	
POF[a]	627	5.13	656	0.33	5.7
TokyoGreen[b,c]	491	8.50	510	0.85	6.2
TokyoMagenta[d,e]	582	11.0	598	0.42	6.8
Napfluorescein[f,g]	595	4.4	660	0.14	8.0

[a]pH = 9. [b]pH = 13. [c]Ref 15. [d]pH = 9. [e]Ref 4. [f]pH = 9. [g]Ref 17.

pH value, the longer-wavelength emission band is observed, probably due to the enhanced acidity of the phenolic proton in the excited state.[16] Whereas a similar phenomenon is also observed in TokyoMagenta, the relative fluorescence intensity for the anion/neutral species is much higher in POF (15/1 at pH = 3) than that in TokyoMagenta (1/1 at pH = 3), corroborating the higher acidity of the phenolic proton in POF in the excited state. At pH = 9, the spectral emission profile is a mirror image of that of the longest-wavelength absorption band with a small Stokes shift of 29 nm ($\Delta\nu$ = 700 cm^{-1}). Notably, this emission wavelength is shifted by 146 nm to longer wavelengths compared to that of the oxygen analogue, TokyoGreen (λ_{em} = 510 nm), and even by 58 nm than that of the silicon analogue, TokyoMagenta (λ_{em} = 598 nm); it is thus comparable to that of Napthofluorescein, which is a highly π-extended analogue of fluorescein.[17] These results demonstrate the substantial impact of the phosphine oxide moiety on the electronic structure. The fluorescence quantum yield of the neutral form of POF (Φ_{FL} = 0.28 at pH = 3) was comparable with that of the anionic form (Φ_{FL} = 0.29 at pH = 9), while the absorption maxima of these species differed significantly from each other ($\Delta\lambda_{abs}$ = 139 nm). These properties suggest potential utility for POF as a pH-responsive fluorescent probe (*vide infra*).‡

It should also be noted that POF showed high resistance to photobleaching. Under irradiation of a Xe lamp (300 W; λ_{ex} > 350 nm), the photostability of POF in a phosphate-buffered solution (pH = 7.4) was compared to those of TokyoGreen and TokyoMagenta at similar concentration (7.4 x 10^{-6} M, Fig. 4a). While 79% of POF remained after irradiation for 30 min, only 11% of TokyoGreen and 2.4% of TokyoMagenta persisted. Even after 2 h of irradiation, 53% of POF remained. Similar photobleaching experiments, using a Xe lamp (300 W) with a

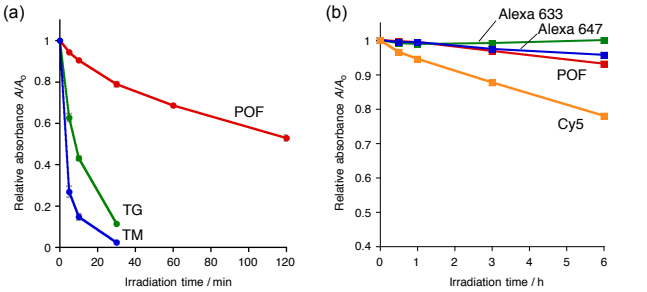

Fig. 4 Photostability of fluorescein dyes in phosphate buffer solutions (pH = 7.4) containing 1% of DMSO. (a) Time-dependent absorption of 7.4 x 10^{-6} M solutions of TokyoGreen (TG, green circles), TokyoMagenta (TM, blue circles), and POF (red circles) upon irradiation with a Xe lamp (300 W; λ_{ex} > 350 nm). (b) Time-dependent absorption variation of solutions of Alexa Fluor 633 (green squares), Alexa Fluor 647 (blue squares), Cy5 (orange squares), and POF (red squares) as a function of irradiation time with a Xe lamp (300 W) using a band-pass filter of 630 ± 10 nm. Solution concentrations were adjusted to be comparable in terms of optical density at 630 nm.

band-pass filter of 630 ± 10 nm, revealed that the photostability of POF exceeds Cy5, and is comparable to those of the representative photostable red-emissive fluorescent probes Alexa Fluor 633 and Alexa Fluor 647 (Fig. 4b). The high resistance of POF towards photobleaching is most likely due to its low-lying HOMO, which may play an important role in the context of diminishing the reactivity towards oxygen in the excited state. These results indicate great potential for POF as a red-emissive fluorescent probe.

As POF was found unable to permeate cell membranes due to its anionic character in the incubation medium, the acetyl-protected derivative AcPOF was prepared for applications in living cells (Scheme S1). Fig. S4 shows HeLa cells (λ_{ex} = 633 nm) stained with of AcPOF (c = 5 μM, t = 10 min, T = 37 °C), which exhibited intense fluorescence within the cells. The cytotoxicity of POF in HeLa cells was evaluated by the MTT assay, and the results revealed no significant cytotoxicity even after incubation with 10 μM of POF for 24 h (cell viability: 84%; Fig. S5). To examine the performance of POF as a ratiometric fluorescent pH probe, we stained RAW 264.7 cells with AcPOF and further incubated the cells in pH calibration buffer solutions (pH = 4.5 or 6.5; Thermo Fisher Scientific), containing

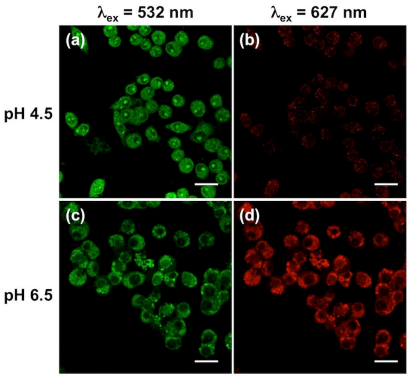

Fig. 5 Fluorescence images of RAW 264.7 cells stained with AcPOF (c = 5 μM, t = 15 min, T = 37 °C). After removal of the media, cells were further incubated in buffered solutions of pH 4.5 (panels a and b) and pH 6.5 (panels c and d) containing 10 μM of valinomycin and 10 μM of nigericin. Images were obtained at an excitation wavelength of 532 nm (panels a and c) or 627 nm (panels b and d). Scale bars represent 20 μm.

valinomycin and nigericin (10 μM each). Upon excitation at 532 nm, which represents the isosbestic point in the excitation spectra (Fig. S2), the intensities of the fluorescence emissions were comparable between pH 4.5 and 6.5 (Figs. 5a and 5c). However, upon excitation at 627 nm, a significant increase in fluorescence intensity was observed for pH 6.5 (Figs. 5b and 5d). These findings suggest that POF should be a promising pH-responsive fluorescent probe under relatively acidic condition.

In summary, we successfully synthesized a phosphine oxide analogue of fluorescein, POF. The electron-withdrawing character of the phosphine oxide moiety endows POF with a significantly red-shifted absorption and fluorescence compared to those of the corresponding oxygen and silicon analogues, and the emission maximum is located in the far red region. The phosphine oxide moiety also enhances the photostability of POF and decreases the pK_a value of the phenolic moiety compared to other relevant fluorescein derivatives. These characteristics should render POF an attractive fluorescent probe for monitoring e.g. intracellular pH values. Further structural modifications of POF directed towards applications in live cell imaging are currently in progress in our laboratory.

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Notes and references

‡As a preliminary study aiming at the utility of POF as a pH-responsive fluorescent probe, we recorded its excitation spectra at an emission wavelength of 720 nm under various pH conditions. The plots of the fluorescence intensity at two distinct excitation wavelengths (480 and 627 nm) as a function of the pH value furnished a pK_a value of 5.7, which is in good agreement with that obtained from the absorption titration (Fig. S3a). The plot of the fluorescence intensity ratio obtained using excitation wavelengths of 532 and 627 nm (I_{627}/I_{532}) could be fitted with a sigmoidal curve (Fig. S3b). This result indicates that pH values in the range 5.1–6.3 can be accurately determined based on a ratiometric analysis using the fluorescence intensities at these excitation wavelengths.

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