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Ultra-sensitive and selective Hg^{2+} chemosensors derived from substituted 8-hydroxyquinoline analogues†

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Novel analogues of 8-hydroxyquinoline with phosphinate or thiophosphinate functions and styryl fluorophores in the *para* position to the nitrogen atom were prepared *via* multi-step syntheses, using phosphorylation and Wittig coupling reactions. A strong affinity between the quinoline analogues and heavy metal ions such as Pb^{2+}, Cd^{2+} and Hg^{2+} was highlighted. The interaction of the metal ions with the nitrogen of the styrylquinoline leads to a large red shift of the absorption and emission spectra in agreement with an increase of the photoinduced charge transfer character of the styryl fluorophore. In the presence of metal ions the appearance of a green fluorescence emission is also observed upon excitation at 420 nm or 840 nm, thanks to a significant increase of the two-photon response. Under optimal conditions, a mercury concentration of 15 ppt in a partially aqueous medium can be detected using the thiophosphinate derivative without interference from other metal ions.

Introduction

The design of sensors for the detection of heavy-metal ions has been a field of continuing research since the control of water resources and natural environments has become a major challenge worldwide.[1] Among heavy metal ions, lead, nickel, cadmium and mercury salts specifically need to be detected,[2] the last two being classified among ''priority hazardous substances'', toxic, persistent and bioaccumulative chemicals. The Hg^{2+} ion is considered to be highly toxic causing environmental and health problems.[1,2] The level of this ion is therefore subjected to strict regulations and should presently not exceed 1 μg L^{-1}.[2] Apart from analytical techniques (atomic absorption or emission spectroscopy and inductively coupled plasma spectroscopy), the methodology based on fluorescent molecular sensors has attracted considerable interest due to the superior sensing sensitivity and selectivity it offers.[3] Some examples described the use of chemodosimeters which have some drawbacks because of their irreversibility.[4] However, among fluorescent molecular sensors currently available, only a few are competitive in terms of sensitivity and selectivity, while direct measurements in aqueous medium remain a crucial issue.[5] The development of sensitive and selective fluorescent sensors is thus a subject of constant interest. In the course of our recent ongoing program directed toward the synthesis of novel fluorophores[6] and on the basis of recent work on 8-hydroxyquinoline (8-HQ) analogues,[7] we designed novel sensors for the detection of Hg^{2+} and wish to describe herein their synthesis, photophysical and complexing properties.

Results and discussion

Synthesis

We chose the 8-hydroxyquinoline moiety as one of the most important chelators of metal ions[8] and anticipated that the introduction of a phosphane oxide or thiophosphinate moiety would enhance the selectivity towards mercury ions. We therefore engaged in the synthesis of thiophosphinates 8-HQ-4-St-PS **4** and 8-HQ-4-St-PO **3** (Scheme 1), the arylvinylidene side chain being selected in order to shift the fluorescence emission in the visible region (as well as excitation in the near UV region) for easier implementation in on-field devices.

The synthesis of model 8HQ-PO **2** was straightforward starting from 8HQ in the presence of sodium hydride and

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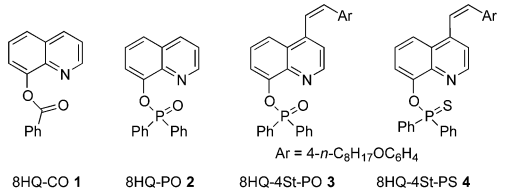
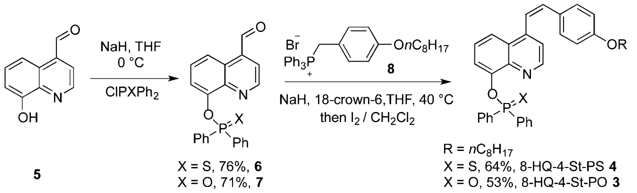


Scheme 1 Hydroxyquinoline fluorescent sensors.



Scheme 2 Synthesis of chemosensors 8-HQ-4-St-PS **3** and 8-HQ-4-St-PO **4**.

diphenylphosphanoyl chloride at 0 °C in THF.[9] The introduction of the donating vinylidene groups required a multistep synthesis starting from the known aldehyde-substituted 8-hydroxyquinoline **5** (Scheme 2).[10] The aldehyde **5** was phosphorylated with diphenylthiophosphanyl chloride[11] in good yield. The corresponding phosphorous derivative **6** was then engaged in a Wittig reaction in the presence of the phosphonium salt **8**.[12] The *Z* isomer[13] 8-HQ-4-St-PS **3** was isolated in 64% yield after an isomerization reaction in the presence of iodine. A similar synthesis was accomplished for the preparation of 8-HQ-4-St-PO **4** and led to the desired sensor in 38% yield over two steps.

Photophysical properties of the ligands

The absorption and fluorescence properties of the quinoline derivatives are collected in Table 1, and their absorption and emission spectra are shown in Fig. 1(a) and (b), respectively. The fluorophores show an absorption band in the UV region. The phosphane oxide derivative and the carbonyl compound have comparable extinction coefficients. As expected the introduction of a styryl derivative in the *para* position of the quinoline derivative induces a noticeable red-shift of the absorption and the emission spectra due to the intramolecular charge transfer character of the transition. As previously described for the carbonyl derivatives,[7a] the proximity of the phosphoryl oxygen (sulfur) lone pair to the acceptor nitrogen of the fluorophores induces a radiationless deactivation process through a $^3(n-\pi)^*$ state.

Table 1 Photophysical properties of quinoline derivatives in CH_3CN. Maxima of the one-photon absorption λ_{abs} [nm] and of steady-state emission λ_{em} (nm), the molar absorption coefficient ε (M^{-1} cm^{-1}), and the fluorescence quantum yield Φ_F

Product	λ_{abs}^{max} (nm)	ε^{max} (M^{-1} cm^{-1})	λ_{em}^{max} (nm)	Φ_F
8HQ	308	2400	393	3.8×10^{-3}
8HQ-CO[a]	275	4700	385	7.6×10^{-4}
8HQ-PO	290	5000	396	2.7×10^{-3}
8HQ-4St-PO	332	7600	461	2.6×10^{-3}
8HQ-4St-PS	342	7600	459	2.6×10^{-3}

[a] Ref. 7*a*.

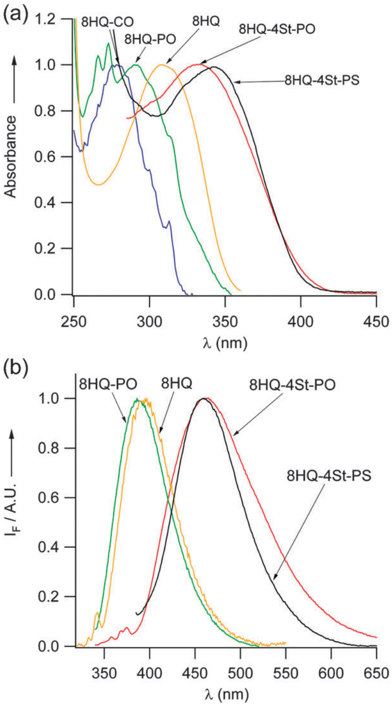


Fig. 1 (a) Absorption of quinoline derivatives in CH_3CN. (b) Corrected normalised emission spectra of the quinoline derivatives in CH_3CN.

Cation-induced photophysical changes

The complexation of the phosphane oxide and the phosphane sulfide derivatives was investigated in the presence of heavy metal ions in acetonitrile. The complexation of mercury salts with the phosphane oxide derivative 8HQ-PO **2** (Fig. 2) was studied in acetonitrile with a ligand concentration of 22 μM upon addition of increasing aliquots of mercury perchlorates. As expected, a red shift of the absorption band from 332 nm to 380 nm occurred, confirming the role of the quinoline nitrogen atom in the coordination of the metal ion.

Therefore, when using an excitation wavelength of 337 nm, an emission band appears at 490 nm and a decrease of the fluorescence is observed for the higher concentrations. The decrease of the fluorescence intensity for larger concentration of mercury can be explained by stronger interaction between the fluorophore and the mercury and by a possible electron transfer process. The analysis of the evolution of the spectra using SPECFIT software revealed that different complexes are formed with the following different stoichiometries 1 : 1 and 1 : 2 (ML and ML_2) (Fig. 2).

The stability constants derived for the phosphane oxide derivatives and the phosphane sulfide derivatives are given in Table 2. For the 8HQ-4St-PO **3** (Fig. 3), the addition of mercury induces a larger photophysical effect in relation to an efficient

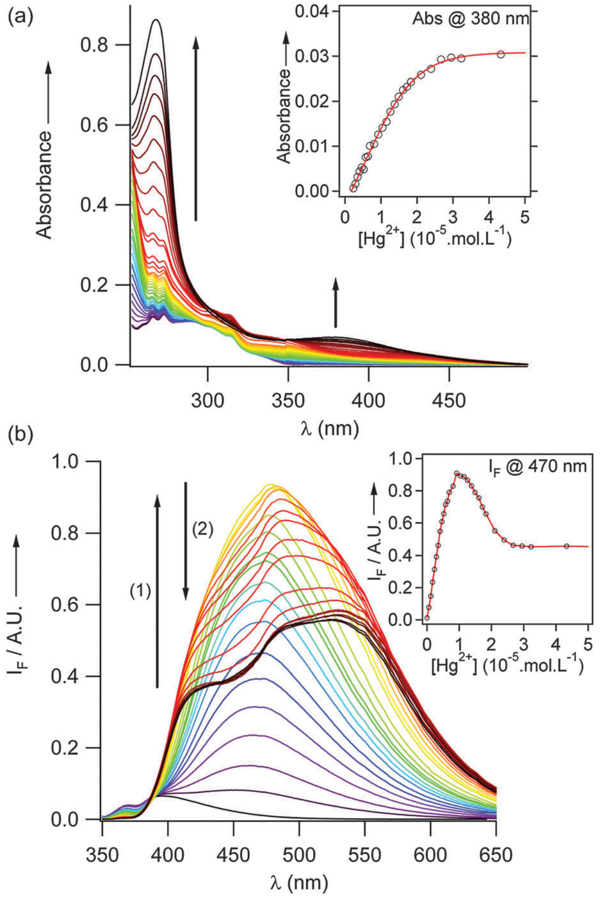


Fig. 2 (a) Absorption and (b) corrected emission spectra of 8HQ-PO **2** in the presence of increasing concentrations of Hg^{2+} in CH_3CN (C_L = 22 μM, λ_{exc} = 335 nm).

Table 2 Stability constants of quinoline derivatives with Pb^{2+}, Cd^{2+} and Hg^{2+} in CH_3CN

	8HQ-CO[a]	8HQ-PO		8HQ-4St-PO	8HQ-4St-PS	
	log β_{ML}	log β_{ML}	log β_{ML2}	log β_{ML}	log β_{ML}	log β_{ML2}
Hg^{2+}	4.5 ± 0.7	7.0 ± 0.2	12.6 ± 0.2	5.8 ± 0.5	7.5 ± 0.5	13.5 ± 0.7
Pb^{2+}	1.2 ± 0.2	6.7 ± 0.2	12.0 ± 0.3	6.3 ± 0.2	4.7 ± 0.1	11.2 ± 0.3
Cd^{2+}	—	—	—	6.6 ± 0.2	5.2 ± 0.2	—

[a] Ref. 7*a*.

photoinduced intramolecular charge transfer phenomenon. The absorption band shifts from 345 nm to 410 nm in the presence of mercury. Direct emission of the complex at 420 nm induces the formation of a non-structured band at 560 nm (Fig. 3). For the phosphane sulfide derivative 8HQ-4St-PS **4** (Fig. 4), similar photophysical effects have been observed upon complexation of heavy metal ions. In comparison to the results obtained by Jiang's group,[7a] the higher stability constants obtained here confirm that the introduction of a phosphane oxide or a phosphane sulfide function increases the ability of the probe to coordinate heavy metal ions. Because of the high affinity of the phosphane sulfide derivative for mercury, the highest complexation constant was observed for the 8HQ-4St-PS **4**. It should be noticed that for compounds **3** and **4** only the 1 : 1 stoichiometry complex is observed because of the steric hindrance due to the incorporation of the styryl conjugated group.

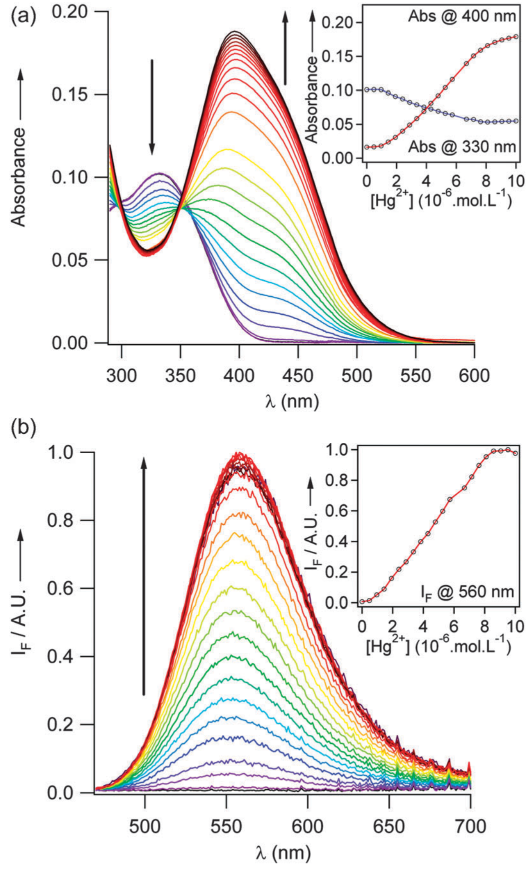


Fig. 3 (a) Absorption and (b) corrected emission spectra of 8HQ-4St-PO **3** in the presence of increasing concentrations of Hg^{2+} in CH_3CN (C_L = 22 μM, λ_{exc} = 420 nm).

Two-photon absorption

In addition to standard one-photon excitation, we also investigated the potential use of two-photon excitation (TPE) for selective excitation of the probe. There has been a lot of efforts in designing bright biphotonic fluorescent probes[14,15] in the last decade mainly driven by the advantages TPE provides for biological imaging.[16] In the field of fluorescent probes, TPE could offer an additional benefit by allowing selective excitation in the presence of fluorescent contaminants. We have investigated the TPE response of the thiophosphinate 8-HQ-4-St-PS **4** for mercury detection, expecting that only the complex would show a significant TPE response in the particular spectral range of interest. This indeed proved to be the case (Fig. 5a), as pure 8-HQ-4-St-PS **4** did not show detectable fluorescence when two-photon excited in the same range. Taking advantage of the large contrast between the TPE response of 8-HQ-4-St-PS **4** and of its mercury complex and of the high sensitivity and affinity for mercury of the 8-HQ-4-St-PS **4**, we further demonstrated that monitoring fluorescence by two-photon excitation at an optimum wavelength (typically in the 800–900 nm range) indeed allowed monitoring Hg^{2+} concentration (Fig. 5b).

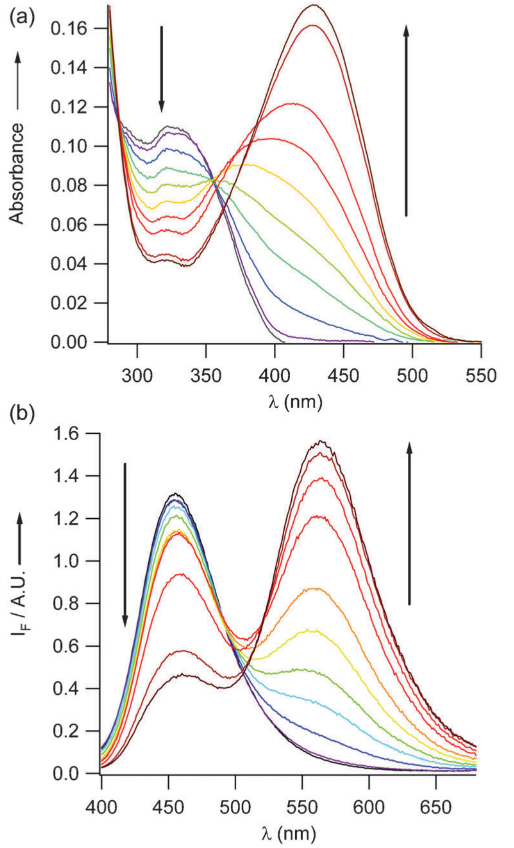


Fig. 4 (a) Absorption and (b) corrected emission spectra of 8HQ-4St-PS **4** in the presence of Hg^{2+} in CH_3CN (C_L = 15 μM, λ_{exc} = 350 nm).

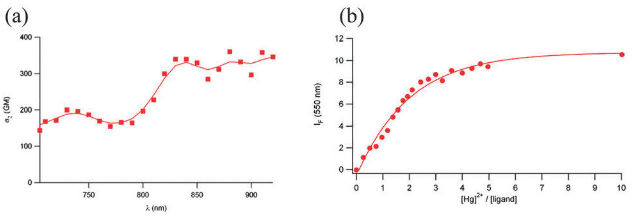
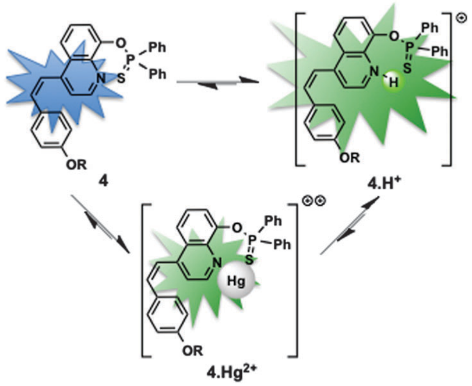


Fig. 5 (a) Two-photon absorption of 8-HQ-4-St-PS **4** in the presence of an excess of Hg^{2+} in CH_3CN ($C_L = 10^{-4}$ M, $C_{Hg(ClO_4)_2} = 10^{-3}$ M). No TPEF signal is observed in the absence of Hg^{2+}. (b) Fluorescence signal of 8-HQ-4St-PS **4** in the presence of increasing concentrations of Hg^{2+} in CH_3CN (two-photon excitation at 840 nm) $C_L = 10^{-4}$ M.



Scheme 3 Equilibrium involved upon complexation with Hg^{2+}.

Practical determination of mercury in water

In view of practical applications, complexation studies were then carried out in a partially aqueous medium of 40/60 CH_3CN–H_2O. Titration experiments show that 8-HQ-4St-PS **4** was able to coordinate mercury in aqueous medium (see ESI†). Analysis of the spectra revealed that under these conditions a 1 : 1 complex is formed with mercury with $\log K_{11} = 3.8 \pm 0.1$. In $CH_3CN : H_2O$ (40 : 60 v/v),[17] the pK_a of 8-HQ-4St-PS **4** was measured to be 3.23 ± 0.02, which is lower than the complexation constant of 8-HQ-4St-PS **4** with mercury. In addition, the quantum yield of the protonated form **4**·H^+ being 10 fold higher than the mercury complex **4**·Hg^{2+}, we optimized the sensing experimental conditions (pH = 3) to exploit the replacement of a proton by mercury to ensure larger fluorescence variations, and consequently improved sensitivity (Scheme 3).[18]

As previously observed, the addition of mercury induced a bathochromic shift of the absorption band, and quenching of the fluorescence was observed in relation to the replacement of the proton by mercury (Fig. 6). Scheme 3 outlines the equilibrium involved upon complexation with mercury under these operating conditions. Taking advantage of this highly efficient quenching process, a calibration curve can be established. The measured detection limit was found to be 0.5 nanomol per liter, and the theoretical detection limit, calculated to be three times the standard deviation of the background/noise ratio, was found to be 0.075 nanomol per liter (15 ppt).

The selectivity of the probe was also tackled. As shown in Fig. 7, no significant change in the fluorescence was observed upon addition of high concentrations of interfering cations such as Li^+, Na^+, Mg^{2+}, K^+, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^+, Cu^{2+}, Zn^{2+}, Ag^+, Cd^{2+} and Pb^{2+} at 1 or 0.1 mM. Moreover, after addition of 1 equiv. of Hg^{2+}, the fluorescence was still quenched immediately, indicating that the presence of other cations does not affect the sensing process and its sensitivity.

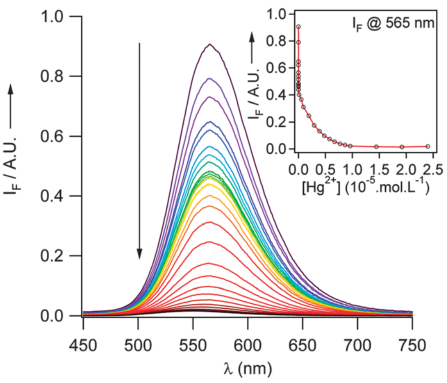


Fig. 6 Corrected emission spectra of 8-HQ-4-St-PS **4** in the presence of increasing concentrations of Hg^{2+} in 40 : 60 v/v CH_3CN–H_2O (C_L = 13 μM, λ_{exc} = 420 nm).

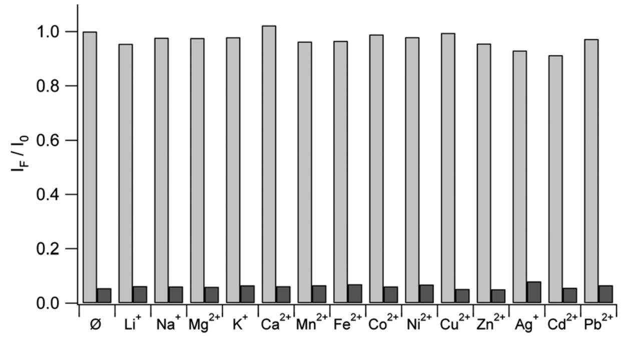


Fig. 7 (light gray) Response I_F/I_0 of 8-HQ-4-St-PS **4** (13 μM) in CH_3CN–H_2O (40 : 60 v/v) at pH = 3 plus interfering ions Li^+, Mg^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+} and Cd^{2+} at 1 mM, and Na^+, K^+, Mn^{2+}, Fe^{2+}, Ni^+, Cu^{2+}, Ag^+ and Pb^{2+} at 0.1 mM (λ_{exc} = 420 nm, λ_{em} = 565 nm). (dark gray) *idem* in the presence of Hg^{2+} (13 μM).

Conclusion

In conclusion, we have synthesized a series of fluorescent chemosensors based on hydroxyquinoline bearing chalcogenic phosphanes such as phosphane oxide and sulfide derivatives. These original derivatives showed an intense absorption band in the near-UV region and a fluorescence emission in the visible region. The large Stokes shift value and the unresolved vibronic structure of the fluorescence spectra strongly support the intramolecular charge transfer (ICT) character of the low-energy transition, which is thus expected to be highly sensitive to complexation by a cationic species. Indeed, addition of a heavy metal cation such as Hg^{2+} induced a bathochromic shift of the absorption and emission spectra thanks to the increase of the electron-withdrawing character of the complexed quinoline moiety. In view of practical application, the cation complexation was studied in a CH_3CN–H_2O mixture under optimized pH conditions (pH = 3) and the presence of mercury cations induced a major fluorescence quenching which allowed reaching a detection limit in the picomolar range (75 pM–15 ppt). This outstandingly low detection limit does therefore meet the requirements defined by the World Health Organization for drinking water. Further efforts will be dedicated to insert the fluorescent sensor in a microfluidic chip.

Experimental

General information: all reactions were performed under a dry argon atmosphere by using standard Schlenk techniques. Column chromatography was performed with E. Merck 0.040–0.063 mm Geduran silica gel. ^1H NMR, ^{13}C NMR and ^{31}P NMR were recorded on Bruker AV 300 and 400 instruments. All signals were expressed as ppm (δ) and internally referenced to residual protio solvent signals. Coupling constants (*J*) are reported in Hz and refer to apparent peak multiplicities. Mass spectrometry analyses were performed at IMAGIF/ICSN and at the University P. et M. Curie. Compounds **5**[10] and **8**[11] were prepared according to published procedures. The preparation of diphenylthiophosphanyl chloride was realized according to the literature.[11]

Syntheses of 8-hydroxyquinoline analogues

Thiophosphinate 6. To a solution of 4-formyl-8-hydroxyquinoline **5** (300 mg, 1.7 mmol) in tetrahydrofuran (10 mL) at 0 °C was added 60% w/w NaH in mineral oil (69 mg, 1.7 mmol). The resulting mixture was stirred for 15 minutes at 0 °C, then diphenylthiophosphinic chloride (429 mg, 1.7 mmol) was added dropwise. The mixture was stirred for 3 h at room temperature. The mixture was quenched with water and extracted with dichloromethane (2 × 20 mL). The organic phase was dried over $MgSO_4$ and the volatiles removed under vacuum. The residue was purified by flash silica gel chromatography (cyclohexane–ethyl acetate 80/20) to give 450 mg (71%) of the product. ^1H NMR (300 MHz, $CDCl_3$): δ = 10.47 (s, 1H), 9.18 (d, 1H, *J* = 4.2 Hz), 8.76 (d, 1H, *J* = 7.3 Hz), 8.22–8.14 (m, 4H), 7.79 (d, 1H, *J* = 4.2 Hz), 7.65–7.56 (m, 1H), 7.51–7.46 (m, 7H) ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 192.6, 150.0, 147.3, 136.5, 135.2, 133.7, 132.1, 131.4 (d, *J* = 11.7 Hz), 129.0, 128.4 (d, *J* = 13.8 Hz), 126.2, 125.2, 121.1 (d, *J* = 5.7 Hz), 120.8. ^{31}P (121.5 MHz, $CDCl_3$): δ = 84.2. HRMS (ESI+): *m/z*: calcd for $C_{90}H_{94}O_4P$: 1269.6884; found: 1269.6937 $[M + H]^+$.

Thiophosphinate 4. To a solution of thiophosphinate **6** (100 mg, 0.25 mmol) and phosphonium salt **8** (154 mg, 0275 mmol) in tetrahydrofuran (1 mL) at 0 °C were added 60% w/w NaH in mineral oil (20 mg, 0.50 mmol) and 18-crown-6 (13.2 mg, 0.05 mmol). The resulting mixture was stirred at room temperature for 2 hours, then quenched with water and extracted with dichloromethane (2 × 10 mL). The organic phase was dried over $MgSO_4$ and the volatiles removed under vacuum. The crude product was redissolved in dichloromethane (5 mL) and a catalytic amount of iodine was added. The solution was stirred at room temperature for 3 h under light exposure then washed with saturated aqueous $Na_2S_2O_3$ and dried over $MgSO_4$. The residue was purified by flash silica gel chromatography (cyclohexane–ethyl acetate 80/20 then 70/30) to give 94 mg (64%) of the product. ^1H NMR (300 MHz, $CDCl_3$): δ = 8.79 (d, 1H, *J* = 3 Hz), 8.20–8.27 (m, 4H), 7.80 (d, 1H, *J* = 6.2 Hz), 7.64 (d, 1H, *J* = 6.2 Hz), 7.40–7.55 (m, 7H), 7.35–7.27 (m, 3H), 6.91 (d, 1H, *J* = 9 Hz), 6.85 (d, 2H, *J* = 12.4 Hz), 6.76 (d, 2H, *J* = 12.4 Hz), 6.60 (d, 1H, *J* = 9 Hz), 3.85 (t, 2H, *J* = 6.6 Hz) 1.65–1.72 (m, 2H), 1.25–1.43 (m, 10H), 0.87 (t, 3H, *J* = 6.7 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 159.8, 149.9, 147.4, 144.4, 135.5, 133.8, 131.8 (d, *J* = 11.4 Hz), 130.4, 128.3 (d, *J* = 13.9 Hz), 128.0, 125.6, 123.2, 121.4, 120.1, 114.1, 67.9, 31.8, 29.3, 29.2, 26.0, 22.6, 14.1. ^{31}P (121.5 MHz, $CDCl_3$): δ = 83.4. HRMS (ESI+): *m/z*: calcd for $C_{39}H_{37}NO_2PS$: 592.2439; found: 592.2449 $[M + H]^+$.

Phosphinate 7. To a solution of 4-formyl-8-hydroxyquinoline **5** (300 mg, 1.7 mmol) in tetrahydrofuran (10 mL) at 0 °C was added 60% w/w NaH in mineral oil (69 mg, 1.7 mmol). The resulting mixture was stirred for 15 minutes at 0 °C, then diphenylphosphinic chloride (402 g, 1.7 mmol) was added dropwise. The mixture was stirred for 3 h at room temperature. The mixture was quenched with water and extracted with dichloromethane (2 × 20 mL). The organic phase was dried over $MgSO_4$ and the volatiles removed under vacuum. The residue was purified by flash silica gel chromatography (cyclohexane–ethyl acetate 80/20) to give 450 mg (71%) of the product. ^1H NMR (300 MHz, $CDCl_3$): δ = 10.49 (s, 1H), 9.27 (d, 1H, *J* = 4.1 Hz), 8.72 (d, 1H, *J* = 8.7 Hz), 8.06–8.14 (m, 4H), 7.92 (dt, 1H, J = 7.7 Hz, J = 1.3 Hz), 7.82 (d, 1H, J = 4.1 Hz), 7.57 (t, 1H, J = 8.5 Hz), 7.53–7.38 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 192.6, 150.3, 132.5, 132.1 (d, J = 10.6 Hz), 129.4, 128.5 (d, J = 13.5 Hz), 126.1, 120.6. ^{31}P (121.5 MHz, $CDCl_3$): δ = 32.1. HRMS (ESI+): m/z: calcd for $C_{90}H_{94}O_4P$: 1269.6884; found: 1269.6937 $[M + H]^+$.

Phosphinate 3. To a solution of phosphinate **7** (100 mg, 0.26 mmol) and phosphonium salt **8** (160 mg, 0286 mmol) in tetrahydrofuran (1 mL) at 0 °C were added 60% w/w NaH in mineral oil (21 mg, 0.52 mmol) and 18-crown-6 (13.7 mg, 0.052 mmol). The resulting mixture was stirred at room temperature for 2 hours, then quenched with water and extracted with dichloromethane (2 × 10 mL). The organic phase was dried over $MgSO_4$ and the volatiles removed under vacuum. The crude product was redissolved in dichloromethane (5 mL) and a catalytic amount of iodine was added. The solution was stirred at room temperature for 3 h under light exposure then washed with saturated aqueous $Na_2S_2O_3$ and dried over $MgSO_4$. The residue was purified by flash silica gel chromatography (cyclohexane–ethyl acetate 80/20 then 70/30) to give 80 mg (53%) of the product. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.88 (d, 1H, J = 3 Hz), 8.11–8.19 (m, 4H), 7.83 (d, 1H, J = 7.7 Hz), 7.77 (d, 1H, J = 8.3 Hz), 7.40–7.65 (m, 8H), 6.89 (d, 2H, J = 8.6 Hz), 6.84 (d, 1H, J = 12.0 Hz), 6.74 (d, 1H, J = 12.0 Hz), 6.59 (d, 2H, J = 8.7 Hz), 3.85 (t, 2H, J = 6.6 Hz) 1.69–1.76 (m, 2H), 1.25–1.44 (m, 10H), 0.87 (t, 3H, J = 6.8 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 158.8, 149.9, 144.7, 133.9, 132.2 (d, J = 10.6 Hz), 130.4, 128.4 (d, J = 13.5 Hz), 127.9, 126.2, 123.1, 121.4 (d, J = 14.9 Hz), 119.9, 114.1, 67.9, 31.8, 29.3, 29.2, 26.0, 22.6, 14.1. ^{31}P (121.5 MHz, $CDCl_3$): δ = 31.8. HRMS (ESI+): m/z: calcd for $C_{25}H_{30}NO_2$: 376.2277; found: 376.2272 $[M\text{-}C_{12}H_8OP]^+$. (ESI–): m/z: calcd for $C_{12}H_{10}O_2P$: 217.0418; found: 217.0423 $[M\text{-}C_{25}H_{28}NO]^-$.

Spectroscopic measurements. UV/Vis absorption spectra were recorded on a Varian Cary5000 spectrophotometer and corrected emission spectra were obtained on a Jobin–Yvon Spex Fluorolog-3 spectrofluorimeter. The fluorescence quantum yields were determined by using quinine sulfate dihydrate in sulfuric acid (0.5 N; $\Phi_F = 0.546^{19}$) as standards. For the emission measurements, the absorbance at the excitation wavelengths was below 0.1 and so the concentrations were below 10^{-5} mol L^{-1}. The complexation constants were determined by global analysis of the evolution of all absorption and/or emission spectra by using the Specfit Global Analysis System V3.0 for 32-bit Windows system. This software uses singular value decomposition and non-linear regression modelling by the Levenberg–Marquardt method.[20]

Two-photon excited fluorescence spectroscopy. Two-photon excited fluorescence spectroscopy was performed using a mode locked Ti:sapphire laser generating 150 fs wide pulses at a 76 MHz rate, with a time-averaged power of several hundreds of mW (Coherent Mira 900 pumped by a 5 W Verdi). The laser light is attenuated using a combination of half-wave plates and a Glan-laser polariser and the excitation power is further controlled using neutral density filters of varying optical density mounted in a computer-controlled filter wheel. After five-fold expansion through two achromatic doublets, the laser beam is focused by a microscope objective (10×, NA 0.25, Olympus, Japan) into a standard 1 cm absorption cuvette containing the sample. The applied average laser power arriving at the sample was between 0.5 and 15 mW, leading to a time-averaged light flux in the focal volume on the order of 0.1–1 mW μm^{-2}. The generated fluorescence is collected in epi-fluorescence mode, through the microscope objective, and reflected by a dichroic mirror (675dcxru, Chroma Technology Corporation, USA). Residual excitation light is removed using a barrier filter (e650-2p, Chroma) and the fluorescence is coupled to a 600 μm multimode fiber by an achromatic doublet. The fiber is connected to a compact CCD-based spectrometer (BTC112-E, B&WTek, USA), which measures the two-photon excited emission spectrum. The emission spectra are corrected for the wavelength-dependence of the detection efficiency using correction factors established through the measurement of reference compounds having known fluorescence emission spectra. Briefly, the set-up allows for the recording of corrected fluorescence emission spectra under multiphoton excitation at variable excitation power and wavelength.

Absolute values for the two-photon excitation action cross sections $\sigma_2\Phi$ were obtained according to the method described by Xu & Webb, using 10^{-4} M fluorescein in 0.01 M NaOH(aq.) as a reference.[21] **4** was dissolved in CH_3CN at a concentration of 3.8×10^{-5} M. The complexation studies were performed by using different concentrations of mercury.

Solvents and salts. Acetonitrile from Aldrich (spectrometric grade) and millipore filtered water (conductivity $< 6 \times 10^8\ \Omega^{-1}\ cm^{-1}$ at 20 °C) were employed as solvents for absorption and fluorescence measurements. Sodium thiocyanate, potassium thiocyanate, lithium perchlorate, magnesium perchlorate, calcium perchlorate, manganese perchlorate, iron perchlorate, cobalt perchlorate, nickel perchlorate, copper perchlorate, zinc perchlorate, silver perchlorate, cadmium perchlorate and lead(II) thiocyanate, from Aldrich or Alfa Aesar, were of the highest quality available and vacuum dried over P_2O_5 prior to use.

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