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Letter

Achieving Selectivity for Phosphate over Pyrophosphate in Ethanol with Iron(III)-Based Fluorescent Probes

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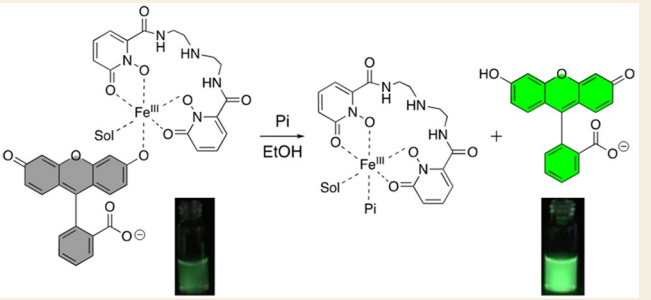
ABSTRACT: Two iron(III)-based molecular receptors employing 1,2-hydroxypyridinone ligands were developed for phosphate recognition and fluorescence sensing via indicator displacement assay (IDA). The tetra- and pentadentate ligands enable anion recognition by the iron(III) center via its remaining one or two open coordination sites. Weak protective coordination of fluorescein at those sites prevents the formation of μ-oxo dimers in aerated solutions. Its rapid and selective displacement by inorganic phosphate results in a 20-fold increase in the fluorescence of the indicator. Both receptors exhibit high affinity for inorganic phosphate and high selectivity over common competing anions, including halides, acetate, carbonate, and, remarkably, pyrophosphate as well as arsenate. Coordination of phosphate to the iron(III) center was confirmed by ATR-IR and ^{31}P NMR spectroscopy.



KEYWORDS: *pyrophosphate, phosphate, iron, fluorescence, supramolecular receptor, indicator displacement assay*

INTRODUCTION

Phosphate is a crucial component of fertilizers needed to maintain the world's food supply. Unfortunately, most of the phosphate used as fertilizer leaches out into surface water, causing widespread eutrophication and hazardous algal blooms. Over 65% of U.S. estuaries and coastal waters now have moderate to severe eutrophication, with significant consequences to the ecology and industry relying on those systems.[1,2] Addressing this issue requires in part facile detection of phosphate in the micromolar range.[3,4] The current protocol of the U.S. Environmental Protection Agency (EPA) for measuring phosphate levels, the molybdenum blue method, relies on the formation of a phosphomolybdate Keggin ion followed by its reduction to yield a blue mixed-valence complex.[5] The slow kinetics of these reactions renders this multistep protocol laborious. Moreover, the strong acidic conditions necessary for the formation of the Keggin ion does not enable distinction between orthophosphate and other polyphosphates such as pyrophosphate that can also be present in large concentration in surface water but have different impacts on algae growth.[6] As such, although much attention has recently been devoted to developing molecular receptors and fluorescent probes for phosphate, effective probes that can readily distinguish between phosphate and pyrophosphate are still needed.[7−12]

Metal complexes are particularly well-suited for probing phosphates by luminescence. Recognition of the anion can be accomplished either allosterically or via direct coordination. As in the case of the heteroditopic ruthenium(II) bipyridyl complexes, allosteric recognition of phosphate is primarily accomplished by directed hydrogen-bonding interactions. Such probes, however, do not work well with aqueous samples and are rarely selective for phosphate, including over pyrophosphate.[13−15] Direct coordination of phosphate is better suited for such applications since the metal ions are able to overcome the high hydration enthalpy of phosphate.[7−11,16,17] The requirements for lability and hardness have limited current studies to copper, zinc, and lanthanide complexes,[18−23,7] some of which have marked selectivity and affinity for phosphate. Unfortunately, although many of those probes are selective for phosphate over competing anions such as bicarbonate and chloride, selectivity for orthophosphate over polyphosphates such as pyrophosphate has not yet been established.

The presence of iron in the active site of many phosphodiesterases and phosphatases suggest that iron could also be used in the design of receptors for phosphate.[24−26] Yet, despite being the most abundant transition metal, iron is rarely explored in the design of molecular receptors, as evidenced by the paucity of iron complexes for anion recognition.[27−30] To the best of our knowledge, no iron-based molecular receptors for any oxyanion that function at neutral pH and that is

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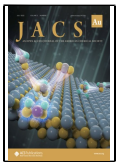

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selective over interfering anions has been reported.[7] Despite its hardness appropriate for hard anions, coordinatively unsaturated iron(III) complexes present several challenges for such applications that are not yet fully mastered. In particular, iron(III) complexes with open coordination sites have a propensity to form μ-oxo dimers,[31,32] which prevents or diminishes further coordination of the targeted anion.[33] The development of Fe^{III}-based receptors for anions thus necessitates a re-engineering of the metal center to prevent such dimerization. In heme-based system, formation of μ-oxo dimers can be prevented by increasing the steric hindrance around the iron center with picket fences[34,35] or via supramolecular assemblies with cyclodextrins.[36] We postulated that, in nonheme iron-based systems, coordination at the open site by a weaker anion could be sufficient to prevent dimerization. Given the propensity of Fe^{III} to quench the fluorescence of organic dyes,[37] such metal-based receptors would also function as a fluorescent probe if this weak anion also fluoresces.

Other parameters should be taken into consideration in the design of the receptor. First, the affinity of receptors for anions are significantly influenced by the overall charge of the metal complex at the pH of interest.[21] Highly negatively charged complexes should be avoided. The Fe^{III} complex must also be sufficiently thermodynamically stable to prevent demetalation. The bioinorganic chemistry of siderophores, natural products that are strong iron chelators,[38] suggest that both of these requirements can be met with tetra- or pentadentate ligands comprising all oxygen donor such as 1,2-hydroxypyridinone (HOPO). In our corresponding molecular receptors Fe^{III}-HOPO-fluo (**1**) and Fe^{III}-HOPO-PhO-fluo (**2**) (Figure 1), the remaining 1 or 2 open coordination sites are protected by fluorescein, a weaker ligand for Fe^{III} than phosphate. We postulated that fluorescein would coordinate sufficiently strongly to iron(III) to prevent formation of μ-oxo dimers, but not too strongly as to enable displacement by phosphate.

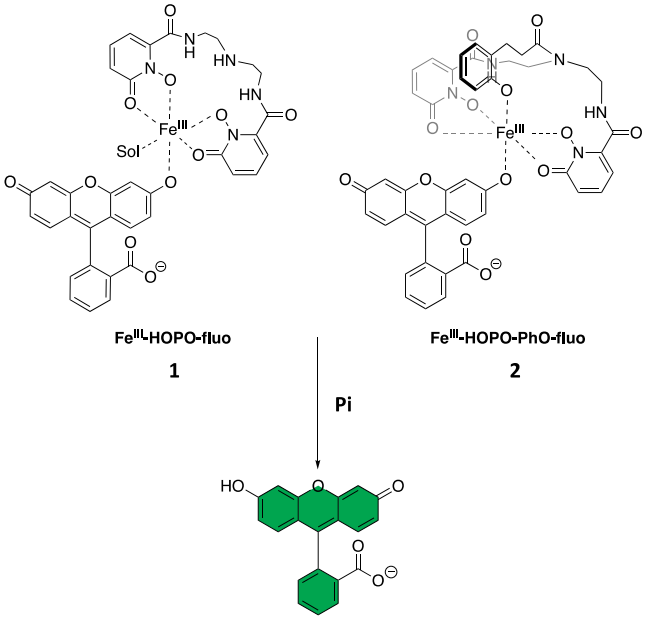
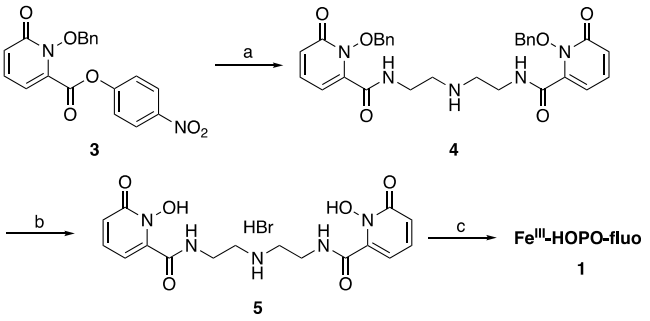


Figure 1. Chemical structures of iron(III)-based luminescent probes for phosphate and detection mechanism. Sol denotes solvent molecules.

RESULTS AND DISCUSSION

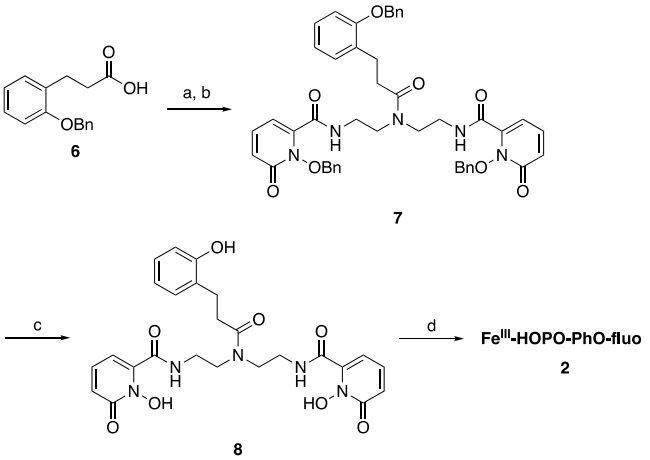
The receptors Fe^{III}-HOPO-fluo and Fe^{III}-HOPO-PhO-fluo were synthesized according to Schemes 1 and 2, respectively.

Scheme 1. Synthesis of Fe^{III}-HOPO-fluo[a]



[a]Experimental conditions: (a) $(NH_2CH_2CH_2)_2NH$, NEt_3, CH_2Cl_2; (b) HBr/AcOH; (c) fluorescein, NaOH(aq), $FeBr_3$, EtOH.

Scheme 2. Synthesis of Fe^{III}-HOPO-PhO-fluo[a]



[a]Experimental conditions: (a) $(COCl)_2$, DMF (cat.), CH_2Cl_2; (b) **4**, NEt_3, CH_2Cl_2; (c) HBr/AcOH; (d) fluorescein, NaOH (aq), $FeBr_3$, EtOH.

The *p*-nitrophenol activated ester of the benzyl-protected HOPO podand **3**, previously synthesized following literature precedence,[39] selectively acylates the primary amino groups of the triamine backbone to yield the protected ligand **4**. Deprotection under strong acidic conditions yields the final ligand **5**, which was further metalated with Fe^{III} in the presence of fluorescein to give the final receptor Fe^{III}-HOPO-fluo.

Fe^{III}-HOPO-PhO-fluo employs a pentadentate ligand whose phenolate moiety occupies one more coordination site of the Fe^{III} center. Activation of the benzyl-protected phenol podand **6**, previously synthesized according to literature reports,[40] with oxalyl chloride enabled coupling to the central secondary amine of **4**, thereby yielding the protected ligand **7**. Deprotection under strong acidic conditions yielded the final ligand **8** that was further metalated with Fe^{III} in the presence of fluorescein to give the final receptor Fe^{III}-HOPO-PhO-fluo, **2**.

In both syntheses, the formation and purity of the ternary complexes **1** and **2** were confirmed by HPLC and ESI-MS (Figures S1, S3, S4, and S6). No μ-oxo diiron dimers were detected, confirming that coordination of the fluorescein ligand is sufficient to protect the Fe^{III} center and prevent the

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formation of bimetallic species. In contrast, in the absence of fluorescein, the μ-oxo diiron dimer is the predominant species observed by MS. The significant line broadening observed in the ^{1}H NMR of the ternary complexes in solution (Figures S2 and S5), which is typical of paramagnetic Fe(III) species, further confirmed coordination of fluorescein to the receptors **1** and **2**. Both Fe^{III}·fluorescein complexes were stable as solids and in ethanol for weeks; both can tolerate up to 10 vol % water with pH adjusted to 7 without significant fluorescein dissociation (<1%) in ethanol.

Direct coordination of phosphate to the iron centers of the receptors concomitant with displacement of the fluorescein moiety upon addition of the oxyanion was first confirmed from attenuated total reflection-infrared (ATR-IR) spectroscopic analysis of the precipitate obtained from Fe^{III}-HOPO-fluo+Pi and Fe^{III}-HOPO-PhO-fluo+Pi. The iron complex Fe^{III}-HOPO-Pi displays the characteristic ν(Fe−O) vibrations at 571 and 461 cm^{-1}, ν(P−O) bands at 1088, 1067, and 968 cm^{-1} and δ (O−P−O) bands at (541) cm^{-1} (Figure 2a).[41−44] Each of those bands was also observed for the Fe^{III}-HOPO-PhO-Pi adduct (Figure S7). These observations are in agreement with the formation of the postulated ternary complexes.

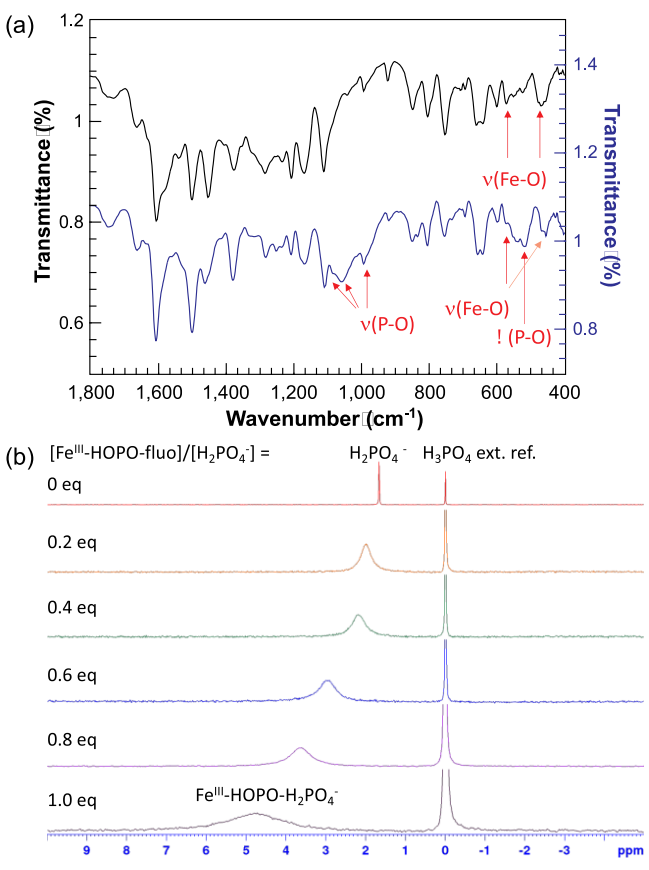

Figure 2. Spectroscopic analyses of Fe^{III}-HOPO-fluo + Pi. (a) ATR-IR spectra of Fe^{III}-HOPO-fluo and Fe^{III}-HOPO-Pi. (b) ^{31}P NMR of $Bu_4N{\cdot}H_2PO_4$* titrated with Fe^{III}-HOPO-fluo (DMSO-d_6, 162 MHz). Experimental conditions: Samples for ATR-IR analysis was prepared by isolating, rinsing, and drying the precipitate formed from Fe^{III}-HOPO-fluo + 1 equiv of Pi. [$Bu_4N{\cdot}H_2PO_4$] = 0.11 M in DMSO-d_6. External reference: 85% H_3PO_4 diluted to 4% with DMSO. *$Bu_4N{\cdot}H_2PO_4$ was used due to the low solubility of inorganic phosphate in DMSO, and low solubility of Fe^{III}-HOPO-Pi in CD_3OD.

Formation of a Fe^{III}L·Pi ternary complex was also supported by NMR spectroscopy. The ^{31}P NMR spectrum of Fe^{III}-HOPO-Pi is nearly featureless (Figure S8), an observation that is attributed to the shortened transverse relaxation times, T_2, of the ^{31}P nucleus by the strongly paramagnetic Fe^{III}. As is apparent in Figure 2b, when referenced to an external standard of H_3PO_4, in a titration monitored by NMR, the ^{31}P signal of phosphate progressively shifts downfield from 1.61 to 4.72 ppm upon gradual addition of Fe^{III}-HOPO-fluo (**1**). This shift is accompanied by a significant line broadening corresponding to a decrease in T_2 of the phosphorus nuclei from 0.11 s (no Fe^{III}-HOPO-fluo) to 1.95 ms (1 equiv of Fe^{III}-HOPO-fluo). Both of those observations are attributable to coordination of orthophosphate to the strongly paramagnetic Fe^{III} center.[45,46] Of note, the presence of a single peak in the ^{31}P also suggests the presence of a rapid equilibrium between bound and free phosphate. Fe^{III}-HOPO-PhO-fluo (**2**), which employs a pentadentate ligand, displays similar behavior with the coordination of phosphate to the Fe^{III} center confirmed from both the ATR-IR and the ^{31}P NMR spectra (Figures S7 and S8, respectively). Unfortunately, further attempts to characterize the ternary phosphate complexes by mass spectrometry were unsuccessful due to the their low solubility and the known ability of phosphate to suppress ionization.[47,48]

The indicator displacement assay (IDA) was evaluated by both UV−visible and fluorescence spectroscopy. A 20-fold turn-on fluorescence was observed upon gradual addition of 1 equiv of orthophosphate (Figure 3a). The fluorescence titrations (Figures 3 and S15) of both receptors were best fitted to a 1:1 binding model from which the equilibrium constants were derived (Table 1). This 1:1 stoichiometry was determined first by evaluating the fit of the titrations and subsequently confirmed by Job plots (Figures S11 and S12 for Fe^{III}-HOPO-fluo and Fe^{III}-HOPO-PhO-fluo, respectively). Interestingly, the use of a tetradentate ligand in Fe^{III}-HOPO-fluo does not appear to favor coordination of two phosphate anions to the metal center. The two receptors display a similar turn-on response (20-fold at 1 equiv) and similar equilibrium constants for phosphate: 8.8×10^5 and 1.1×10^6 M^{-1} for Fe^{III}-HOPO-fluo and Fe^{III}-HOPO-PhO-fluo, respectively. This similarity in both turn-on response and apparent equilibrium constants could be attributed to the comparable core structure of both receptors. Interestingly, the extra phenolate podand of **2** does not appear to affect displacement of the fluorescein moiety by phosphate. A likely coordinated solvent molecule appears to have a similar effect.

The limit of detection (LOD) of phosphate by the two Fe^{III} receptors, commonly estimated as three times the standard deviation of measurement (3σ), are 3.5 and 4.1 μM for Fe^{III}-HOPO-fluo (**1**) and Fe^{III}-HOPO-PhO-fluo (**2**), respectively (Table S1). Although not as sensitive as prior Eu^{III} probes previously developed by our group,[21,22] these iron receptors are sensitive enough to detect problematic phosphate levels in eutrophic samples (2−10 μM).[49,50]

The selectivity of the two iron receptors for phosphate over competing anions commonly found in environmental samples was also evaluated by fluorescence spectroscopy. As shown in the white bars of Figure 4, the fluorescence intensity of both probes is not affected by the addition of 1 equiv of common competing anions including halides, sulfate, and nitrate. Subsequent addition of 1 equiv of phosphate restores the luminescence of the indicator (Figure 4, gray bars), further indicating that these competing anions do not interfere with

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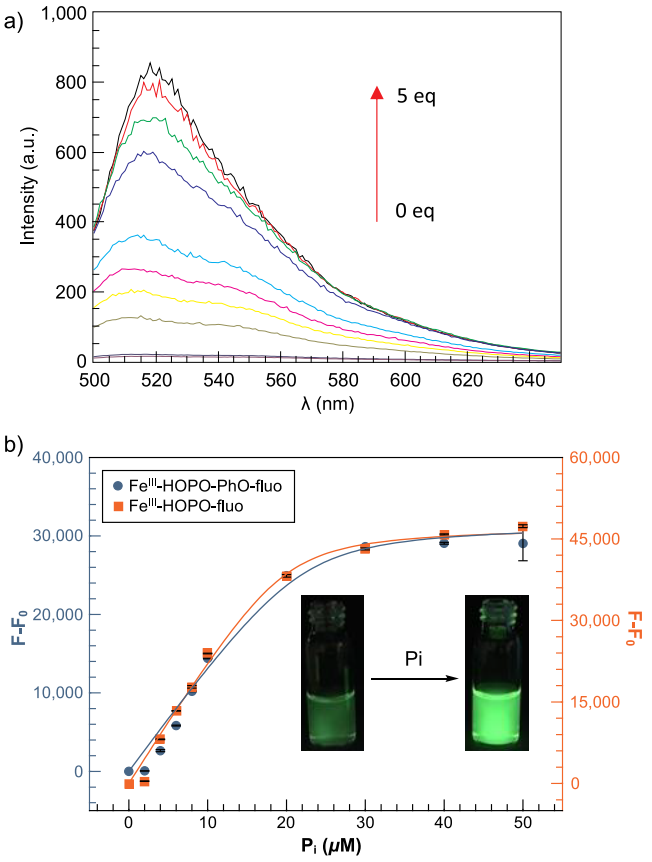

Figure 3. Fluorescence titration of Fe^{III}-HOPO-fluo and Fe^{III}-HOPO-PhO-fluo with phosphate (Pi): (a) fluorescence spectra of Fe^{III}-HOPO-fluo with phosphate. (b) Increase in emission intensity. Experimental conditions: [Fe^{III}-HOPO-PhO-fluo] and [Fe^{III}-HOPO-fluo] = 10 μM in wet ethanol. pH = 7. $\lambda_{excitation}$ = 456 nm, excitation and emission slit widths = 5 nm, voltage = 600 V. T = 25 °C. F = integrated fluorescence intensity from 500 to 650 nm in the presence of anions, F_o = integrated fluorescence intensity in the absence of anions. Fluorescence spectra were obtained 5 min after mixing to ensure that thermodynamic equilibrium was reached. The pH of all solutions was adjusted to 7 carefully by addition of either HCl or NaOH, as necessary.

Table 1. Apparent Equilibrium Constants of Fe^{III}-HOPO-fluo (1) and Fe^{III}-HOPO-PhO-fluo (2) with Orthophosphate

	K_a (M^{-1})
Fe^{III}-HOPO-fluo	8.8 ± 3.4 × 10^5
Fe^{III}-HOPO-PhO-fluo	1.1 ± 0.5 × 10^6

detection of phosphate. Interestingly, Fe^{III}-HOPO-fluo is more selective over bicarbonate and acetate than Fe^{III}-HOPO-PhO-fluo. A more sterically hindered recognition site therefore does not appear to generate higher selectivity for the targeted anion.

Uniquely, and importantly, both Fe^{III}-HOPO-fluo (**1**) and Fe^{III}-HOPO-PhO-fluo (**2**) are selective for phosphate over pyrophosphate. Whereas numerous probes selective for pyrophosphate over phosphates have been described in the literature,[39−41] to the best of our knowledge, complexes **1** and **2** are unique in their reverse selectivity for phosphate over pyrophosphate. This selectivity likely stems from the preferred bidentate binding mode of pyrophosphate and likely steric hindrance at the coordination site.[51,52] Since only one

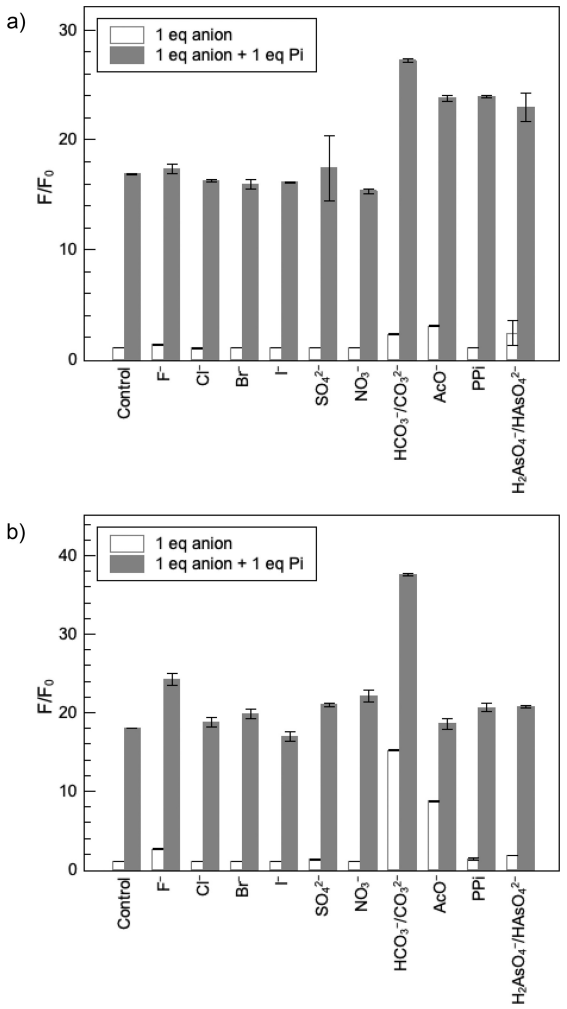

Figure 4. Fluorescence response of (a) Fe^{III}-HOPO-fluo and (b) Fe^{III}-HOPO-PhO-fluo to competing anions. White bars represent the relative fluorescence intensity after addition of 1 equiv of the appropriate anions (NaF, NaCl, NaBr, NaI, Na_2SO_4, $NaNO_3$, $NaHCO_3$, NaOAc, $Na_4P_2O_7$, and $Na_2HAsO_4{\cdot}7H_2O$). Gray bars represent the relative fluorescence intensity after subsequent addition of 1 equiv of phosphate (Pi). PPi denotes pyrophosphate. Experimental conditions: [Fe^{III}-HOPO-fluo] = 10 μM in wet ethanol, pH 7, $\lambda_{excitation}$ = 456 nm, excitation and emission slit widths = 5 nm, F = integrated fluorescence intensity from 500 to 650 nm in the presence of anions, F_o = integrated fluorescence intensity in the absence of anions. T = 25 °C. The pH of all solutions was adjusted to 7 carefully using 0.01 N HCl and 0.01N NaOH. Fluorescence spectra were obtained 5 min after mixing to ensure that thermodynamic equilibrium was reached. Control denotes the same volume of water was used in replacement of anions.

displaceable fluorescein is present, bidentate binding is disfavored. The slightly softer anion, arsenate, also does not displace fluorescein despite its structural similarity to phosphate. This is an unusual selectivity given that most metal probes for phosphate also respond to arsenate.[7] As such, these fluorescent iron(III) probes offer a distinctive ability to rapidly monitor the level of the most important phosphorus species causing nutrient pollution in surface water: phosphate.

CONCLUSION

We describe two nonheme iron(III) complexes, Fe^{III}-HOPO-fluo and Fe^{III}-HOPO-PhO-fluo, for selective recognition of

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inorganic phosphate via indicator displacement assay. In both cases, the open coordination sites were sufficiently protected by weakly coordinating fluorescein to prevent dimerization in aerated solutions. Coordination of inorganic phosphate concomitant with displacement of the fluorescein moiety increases the emission of the latter by 20-fold. Uniquely, these probes distinguish themselves from other receptors that function by direct metal coordination in that they are highly selective for phosphate over pyrophosphate. They are also highly selective over common competing endogenous anions such as carbonate, nitrate, sulfate, halides, and, unusually, arsenate. The limit of detection of the iron(III) receptors, 3.5 and 4.1 μM for Fe^{III}-HOPO-fluo and Fe^{III}-HOPO-PhO-fluo, respectively, enables detection of phosphate typical of eutrophic water samples. On this basis, the two iron(III) probes enable rapid and facile detection of phosphate in eutrophic samples. To the best of our knowledge, these are the first examples employing nonheme Fe^{III}-based molecular receptors for anions. These results thus also provide a blueprint for the development of inorganic phosphate probes that use iron, an earth abundant and economical element.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.2c00200.

Experimental details and characterization data, including ^{1}H and ^{13}C NMR spectra of intermediates and final ligands, ESI-MS data of the intermediates, ligands, and Fe^{III} complexes, HPLC chromatograms of the ligands and Fe^{III} complexes, ultraviolet−visible spectra, fluorescence spectra, and ATR-IR spectra of the Fe^{III} complexes in the absence and presence of phosphate, Job plots of the Fe^{III} complexes with phosphate (PDF)

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Notes

The authors declare no competing financial interest.

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