Revealing the Hydrolysis Mechanism of a Hg$^{2+}$-Reactive Fluorescein Probe: Novel Insights on Thionocarbonated Dyes

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ABSTRACT: As one of the most toxic metal pollutants, mercury is the subject of extensive research to improve current detection strategies, notably to develop sensitive, selective, fast, and affordable Hg$^{2+}$-responsive fluorescent probes. Comprehending the sensing mechanism of these molecules is a crucial step in their design and optimization of their performance. Herein, a new fluorescein-based thionocarbonate-appended Hg$^{2+}$-sensitive probe was synthesized to study the hydrolysis reactions involved in the sensing process. Autohydrolysis was revealed as a significant component of the signal generation mechanism, occurring concurrently with Hg$^{2+}$-catalyzed hydrolysis. This knowledge was used to investigate the effects of key experimental conditions (pH, temperature, chloride ions) on sensing efficiency. Overall, the chemical and physical properties of this new thionocarbonated dye and the insights into its sensing mechanism will be instrumental in designing reliable and effective portable sensing strategies for mercury and other heavy metals.

INTRODUCTION

Among heavy metal ion pollutants, mercury is one of the most known and well studied due to its highly toxic nature and considerable presence in the environment. ¹,² More precisely, its ability to bioaccumulate and biomagnify through the food chain threatens human health by causing damaging effects mainly on the respiratory, endocrine, and central nervous systems.³,⁴ For these reasons, the United States Environment Protection Agency (EPA) has set the maximum tolerable level of mercury contamination in drinking water at 2 ppb (10 nM).⁵ Other governmental agencies have set stricter recommendations, such as Health Canada, which endorses a tolerable level of 1 ppb (5 nM) in drinking water, equivalent to the World Health Organization (WHO) guidelines.⁶,⁷ Therefore, the efficient quantification of mercury in biological and environmental samples is critical to monitor environmental contamination, mitigate risks, assist the diagnosis of disorders, and amend mercury policies.⁸

Traditional analytical techniques such as atomic absorption—emission spectrometry⁹,¹⁰ and inductively coupled plasma mass spectrometry¹¹,¹² are commonly used for the quantification of mercury species with high reliability thanks to their sensitivity and accuracy. However, such sophisticated instrumentation is costly, bulky, not easily amenable to in-field use, and requires qualified personnel to ensure efficient operation. Thus, in the past several years, there has been growing interest in low-priced, rapid, and miniaturized detection methods suitable for real-time and field-deployable sensing of heavy metals. Among alternative strategies available, optical probes—and notably those based on colorimetry and fluorimetry—stand out thanks to their fast response, relatively low cost, and adequate selectivity, as well as their compatibility with simple, compact, and robust instrumentation platforms.¹³–¹⁵ For instance, many rhodamine-based probes have been developed for selective Hg$^{2+}$ sensing through coordination chemistry or Hg$^{2+}$-mediated chemical reactions, generating changes in the absorbance and fluorescence of the dye.¹⁶,¹⁷ Even though their development is promising for real-life applications, scientific advances are still required to make them easier to synthesize, faster, more sensitive, reusable, and water-friendly in the prospect of achieving eco-friendly and efficient probes.

Recently, molecular probes with a thionocarbonate moiety as the recognition receptor were reported for the detection of Hg$^{2+}$. The strategy exploits the inhibition and recovery of intramolecular charge transfer (ICT) in fluorophores through derivatization of their hydroxyl group with a thionocarbonate (Scheme 1). The interaction of the latter with Hg$^{2+}$ induces a hydrolysis process, releasing the alcohol-appended dye. This approach has been reported with fluorescent dye families with distinct photophysical properties such as resorufins,¹⁸,¹⁹ 7-hydroxy-4-methylcoumarin,²⁰ 2-(2’-hydroxyphenyl)benzothiazole,²¹ N-butyl-4-hydroxy-1,8-naphthalimide,²² and seminaphthorhodafluor.²³ The facile permutation of the dye moiety within a common sensing mechanism allows adapting the dye characteristics to the application at hand. Furthermore,
the reported structures operate in aqueous media and have good selectivity toward Hg$^{2+}$. Although the limits of detection (LOD) reported in Scheme 1 are higher than for conventional lab-based techniques, many of them remain below the maximum tolerable levels described previously, and examples of application in real samples such as natural water$^{20,21}$ and living cells$^{19,23}$ have been reported recently, demonstrating the potential of this thionocarbonated family for in-field and real-time sensing platforms. Despite the promise of these thionocarbonate-based dyes, previous studies have only described the sensing mechanism as a Hg$^{2+}$-induced hydrolysis process, without examining the role of autohydrolysis in the signal generation process. Yet, because autohydrolysis is an intrinsic attribute of the thionocarbonate moiety that can negatively impact the LODs achieved, understanding the role of Hg$^{2+}$ in the hydrolysis process is imperative in allowing the development of robust and reliable probes, with a clear understanding of their limitations and how to overcome them.

In the present work, an investigation of the hydrolytic processes observed in thionocarbonate-based probes is described using a customized fluorescein-based compound. Fluorescein, a member of the xanthene class like semi-naphthorhodafluor, is a commercially available and inexpensive fluorophore offering a very high molar absorptivity coefficient and fluorescence quantum yield.$^{24}$ The dye is available both as a sodium salt and as an acid, with the latter bearing hydroxyl...
Examination of the compound commercially available hydrolyses. Furthermore, the spectral properties of hydroxyethyl)piperazine-N− measured by UV−form,32 making two phenolic segments available for reacting dichloromethane, the equilibrium leans toward the lactone forms. In nonpolar and mildly polar solvents such as as a tautomeric equilibrium between the quinoid and lactone intermediate compound.

**RESULTS AND DISCUSSION**

**Synthesis and General Characteristics.** As shown in Scheme 3, compound 1 can be readily prepared in a single step with a high yield (93%). Compared to similar molecular probes reported with a carbonothioate moiety, no preparatory synthesis was required to generate an alcohol-appended fluorophore thanks to the commercial availability of fluorescein acid and o-phenyl chlorothionoformate, avoiding time-consuming and costly synthetic procedures.

The absorption properties of the new carbonothioate-bearing compound compared to that of its commercial precursor can be seen by the naked eye. Fluorescein is bright red in the solid state, whereas 1 is crystalline white (Figure 1). This characteristic color of the commercial acid is due to the stable delocalized quinoid form leading to strong absorption in the visible region. In solution, however, the compound exists as a tautomeric equilibrium between the quinoid and lactone forms. In nonpolar and mildly polar solvents such as dichloromethane, the equilibrium leans toward the lactone form, making two phenolic segments available for reacting with o-phenyl chlorothionoformate to form compound 1. The resulting product preserves the spirolactone conformation in its backbone, preventing the delocalization of electrons along the xanthene moiety, thus leading to low molar absorptivity in the visible region.13C nuclear magnetic resonance (NMR) supports this hypothesis by displaying a signal at 81.1 ppm, distinctive of the sp3-hybridized carbon atom of a spirolactone (Figure S2). Furthermore, the spectral properties of 1 were measured by UV−visible spectrophotometry in an N-(2-hydroxyethyl)piperazine-N′-ethanesulfonic acid (HEPES) buffer solution (20 mM, pH 7.4) containing 1% EtOH to increase solubility. As presented in Figure 1, the molecule indeed shows no distinct absorption band in the visible region due to the lack of delocalization along its backbone. In contrast, upon the addition of 2 equiv of Hg2+, a band at 490 nm appears with a shoulder at around 455 nm, which is characteristic of quinoid fluorescein. These observations suggest the hydrolysis of compound 1 to yield the free fluorescein structure in the presence of Hg2+.

**Fluorescence Response of 1 Toward Hydrolysis.** The emission spectrum of 1 was obtained in a HEPES buffer solution (20 mM, pH 7.4, 1% EtOH). As expected, the thionocarbonate-appended fluorescein alone shows minimal fluorescence when excited at the absorption maximum of fluorescein (λ_{exc} = 490 nm). However, the fluorescence band centered at 516 nm increases over time, reaching a ~7-fold increase in intensity at λ_{em} after 90 min (Figure 2A). Although not reported previously for thionocarbonate-based mercury sensors, the sulfur-rich carbonate is prone to hydrolysis even in the absence of cations. Thus, compound 1’s spontaneous reaction with water is not unexpected, and observation of the latter provides additional insight pertaining to the mechanisms behind the release of the alcohol-exposed fluorophore upon Hg2+ sensing. It is worth mentioning that the awareness acquired on the importance of autohydrolysis can also be applied to other metal ion probes exploiting hydrolysis in their detection mechanism, such as Cu2+.34,35

When 2 equiv of Hg2+ are added to the reaction medium, fluorescein emission at 516 nm becomes clearly distinguishable as early as 5 min following Hg2+ addition, reaching an ~80-fold increase after 90 min (Figure 2B). Although not thoroughly discussed in previous reports, the lack of a plateau within 10 min of Hg2+ addition has been noted as a common feature of thionocarbonate-based probes. The relatively longer comple-

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**Scheme 3. Synthesis of Probe 1**

![Scheme 3](image354x501to534x643)

**Figure 1.** Absorption spectra of 1 (5 μM) in a HEPES buffer solution (20 mM, pH 7.4, 1% EtOH) and 60 min after the addition of 2 equiv of Hg2+. The spectrum of commercial fluorescein acid in the same conditions is shown as a reference. Inset: photographs of 1 and of the commercial precursor in their solid state.
Whereas thiamine determination via oxidation to fluoroimetric reaction-rate method has been reported for the ICT of quinoid fluorescein can be recovered. A good way to skirt the long reaction time of the former’s response kinetically at the need to jettison two carbonothioate moieties before the autohydrolysis of compound 1+Hg2+. Figure 2 shows the mass spectra obtained 5 min after solution preparation. The spectrum without Hg2+ shows a major peak at m/z = 605.0706 attributed to probe 1. A significant peak is also observed at m/z = 469.0725, which agrees with a single-fold hydrolysis of probe 1’s thionocarbonate moiety to yield a hydroxyl-bearing derivative (compound 2, Scheme 4). Furthermore, traces of fluorescein can be identified at m/z = 330.0746. These signals support the autohydrolysis of 1 by a two-step process, occurring as soon as 5 min after the aqueous solution is prepared. The spectrum of 1 taken 5 min after adding 2 equiv of Hg2+ shows the same main peaks but in different proportions. Qualitatively, the signal ascribed to fluorescein is more intense than in the case of compound 2. This suggests that exposition to Hg2+ favors the same chemical process (i.e., hydrolysis) but leads to the final fluorescein product faster. This is coherent with the fluorescence data shown previously for both the free and Hg2+-exposed probes, where the fluorescence rate is faster for the latter.

The specific role of Hg2+ as a facilitator for the hydrolysis is explained by the cation’s soft Lewis acidity. Indeed, the coordination of mercury to sulfur increases the electrophilicity of the carbon atom of the C=S bond, thus making the site more reactive toward hydrolysis and accelerating the process. This catalytic activity is supported by a study showing that alkali metal ions, thanks to their Lewis acidity, may catalyze the ethanolation of substituted p-phenyl thionocarbonates. Moreover, the efficiency of the assisted hydrolysis follows Pearson’s qualitative HSAB concept, with soft acids allowing to coordinate a soft C=S site to catalyze the nucleophilic attack. The importance of the nature of the electrophilic center was confirmed by synthesizing the analogous dicarbonothioate fluorescein ester (compound 3, Scheme 5) and assessing its relative fluorescence behavior at 516 nm. For instance, the fluorescence rate is faster for the fluorescein product faster. This is coherent with the fluorescence rate faster for the latter.

| Probe | 1 | 2 | 2/1 | 3 |
|-------|---|---|-----|---|
| I0f/I0probe | 7 | 12 | | |
| I0f/I0probe+Hg2+ | 79 | 45 | | |
| rfluorescein (s⁻¹) | 74 | 645 | 8.7 | 39 |
| rfluorescein+Hg2+ (s⁻¹) | 1501 | 5480 | 3.7 | 44 |
| rfluorescein+Hg2+/rfluorescein | 20 | 8 | | |

“Conditions: 1 µM of the probe in a HEPES buffer solution (20 mM, pH 7.4, 1% EtOH) at 25 °C with and without 2 equiv of Hg2+. I0 and I0f denote the fluorescence intensities at 0 and 90 min, respectively, whereas r designates the fluorescent kinetic rate for 1–3.”
Hg\(^{2+}\)-assisted hydrolytic reactivity. Table 1 and Figure S7 show that 3 has behavior in water similar to 1 with an autohydrolysis rate of 38 s\(^{-1}\). However, 3 does not show significant enhancement in its fluorescence rate upon the addition of Hg\(^{2+}\), indicating that the latter has minimal effect on the hydrolysis process. This experiment confirms that the soft sulfurated coordination site is crucial in ensuring the binding of bivalent mercury ions and the subsequent accelerated hydrolysis of probe 1.

On the other hand, the soft acid–base condition for Hg sensing of this system also explains the selectivity toward Hg usually reported for thionocarbonated probes. The hydrolysis rate of probe 1 was measured in the presence of various metal ions. As seen in Figure 4, the process is quite specific to Hg\(^{2+}\) across a series of 20 other cations tested in equimolar amounts. Most species do not generate a response exceeding the probe’s autohydrolysis. Exceptions to this trend are Ag\(^{+}\) and Au\(^{3+}\), which, although not always tested in reported thionocarbonate-based Hg\(^{2+}\) sensing probes, are known to be thiophilic.\(^{40}\) This positive correlation between thiophilicity and reactivity with probe 1 supports the importance of this parameter on the design of cation-sensitive molecular probes operating on the principle of catalyzed hydrolysis. For environmental detection purposes, the probe’s sensitivity

**Scheme 4. Consecutive Second-Order Reactions Occurring upon Hydrolysis of Compound 1, Where \(k_1\) and \(k_2\) Represent the Respective Reaction Rates**

![Scheme 4](image)

**Figure 3.** Mass spectra of a 45 μM solution of 1 in water (pH 7.4, containing 10% EtOH) and with 2 equiv of Hg\(^{2+}\). Spectra were recorded 5 min after sample preparation and normalized to the signal at \(m/z = 605.0706\).

**Figure 4.** Relative reaction kinetics of 1 in the presence of 2 μM of various ions. Conditions: 1 μM of 1 in a HEPES buffer solution (20 mM, pH 7.4, 1% EtOH) at 25 °C. Error bars represent RSD (\(n = 2\)).

**Scheme 5. Synthesis of Compound 3**

![Scheme 5](image)
toward Ag⁺ and Au³⁺ is not a serious issue as these ions are seldom found at relevant concentrations in natural waters. An interference experiment was also performed and showed a negligible effect of excess amounts of most metallic cations on the probe’s kinetic response toward Hg²⁺ (Figure S8).

Effect of Sample Matrix on the Hydrolysis Behavior of 1. To deepen our understanding of the hydrolysis processes pertaining to the Hg²⁺ sensing ability of compound 1, the effect of temperature, pH, and chloride ions was investigated. In particular, conditions that minimize the spontaneous hydrolysis of 1 while maintaining high sensitivity in Hg²⁺-rich matrices are expected to result in an optimal signal-to-noise ratio and thus lower detection limits.

Figure 5 summarizes the effect of temperature in the range of 20–70 °C on the free probe and in the presence of 2 equiv of Hg²⁺. The fluorescence intensity of 1 increases faster at higher temperatures, regardless of the presence of Hg²⁺. However, the temperature does not affect the reaction rates of auto- and Hg-mediated hydrolyses identically. Although the measured rate in this study is not the reaction rate as defined in Arrhenius’ law, plotting the natural log of fluorescence rate r against 1/T for both Hg²⁺-rich and Hg²⁺-poor samples provides slopes representative of the magnitude of the activation energies for both pathways (Figure S8B). The lesser slope for the Hg²⁺-rich system suggests lower overall activation energy, agreeing with the cation’s role as a catalyst in the hydrolysis process. From the point of view of Hg²⁺ sensing efficiency, these results also allow us to determine an optimal temperature range. The hydrolysis rate ratio between the Hg²⁺-reacted probe and its free form (r₁₀₉/H₂O/r₁) decreases from 11 at 20 °C to 3 at higher temperatures due to a higher autohydrolysis rate (Figure S5C). Therefore, further experiments were performed at 25 ± 1 °C, i.e., typical of room-temperature conditions in most settings.

The effect of pH on the sensing performance of probe 1 was also investigated. The starting hypothesis was that the sensor would be affected not only by pH due to the intrinsic pH sensitivity of the chosen dye but also by pH due to the amount of hydroxide ions available for the hydrolysis process. Figure 6A shows the normalized reaction rate r of probe 1 in the pH 3–10 range in Hg²⁺-free and Hg²⁺-rich media. The first observation is a slow fluorescence increase at low pH values in both media. As fluorescein is the emissive product from probe 1’s hydrolysis, this observation is due to the intrinsic pH-dependent optical properties of the dye. The neutral quinoid form of fluorescein prevalent in the pH 3–4 range has a low absorption cross section at 490 nm (εₑₙₑutral = 2700 M⁻¹ cm⁻¹) and a low quantum yield (Φₑₙₑutral = 29%). In addition, as was discussed previously, to being in equilibrium with its nonfluorescent spirolactone form (Scheme S1). In the pH 5–10 range, the higher molar absorptivity ε and fluorescence quantum yield Φ of the monoanionic and dianionic species (εᵦₑ₃₆₉₉ = 16 500 M⁻¹ cm⁻¹, Φᵦₑ₃₆₉₉ = 36% and εᵦₑ₉₉₈ = 88 000 M⁻¹ cm⁻¹, Φᵦₑ₉₉₈ = 93%, respectively) lead to vastly increased emission intensity. The excitation–emission spectrum of probe 1 in the presence of 2 equiv of Hg²⁺ was recorded between pH 3 and 10, and the results shown in Figure 6B are in accordance with those reported by Slavik in 1994. At pH 3, the excitation band with a maximum at 437 nm is characteristic of the cationic and neutral forms of fluorescein. Spectral features observed between pH 4 and 5 are indicative of the deprotonation equilibria involving the monoanionic form. A drastic change in the spectral profile occurs at pH 6, with the intense fluorescence band at 490 nm characteristic of the highly emissive dianionic structure of fluorescein. Thus, the pH dependence of fluorescein emission can be ascribed as the reason for the lower r measured below pH 7 in Figure 6A. This explanation is valid for cases with and without Hg²⁺ ions, since pH affects the properties of the fluorescein product independently of the hydrolysis process (Figure S9A). Possible effects of pH on the mechanism itself may be concealed by the features discussed above.

A decrease in the hydrolysis rate of probe 1 with 2 equiv of Hg²⁺ is observed at pH ≥ 7 (Figure 6A). This observation cannot be attributed to the pH-dependent properties of fluorescein as the latter exists only as the highly emissive dianionic form above pH ≈ 8. The excitation–emission spectra shown in Figure 6C suggest that the overall intensity decreases without changes to the dianionic structure. The most plausible explanation to this decrease in the rate of hydrolysis is the accessibility of Hg²⁺ ions, as mercury speciation data dictates

Figure 5. (A) Normalized reaction kinetics r of 1, (B) variation of ln r, and (C) hydrolysis rate ratio according to temperature. Conditions: 1 μM of 1 in a HEPES buffer solution (20 mM, pH 7.4, 1% EtOH). Error bars represent RSD (n = 3).
that Hg(OH)$_2$ is the dominant species in alkaline media. The curve shown for probe 1 without mercury (probe 1 only; Figure 6A) supports this hypothesis and suggests that hydrolysis, in this case, is enhanced by the increase in hydroxide ion concentration (Figure S9B). The same trend has been reported recently for other thionocarbonate-based Hg$^{2+}$-responsive probes, but the authors did not suggest Hg$^{2+}$ speciation as a possible cause for the loss of sensing efficiency. Consequently, all following experiments were performed in a HEPES buffer solution at pH 7.4, where mercury ions are deemed dissolved at the concentration employed.

Interference from chloride, a major ionic component of real-life samples (e.g., natural surface waters) was also studied since Hg tends to form stable chloride complexes that could affect the metal's efficiency as a Lewis acid in sensing experiments with probe 1. The presence of sodium chloride in the sample matrix was found to negatively affect the probe’s response at concentrations greater than 1 mM (Figure S10A). This is in accordance with the speciation diagram for chloride complexes of bivalent mercury in aqueous solutions, where the predominant species at pH 7 with ~1 mM NaCl is a soluble HgCl$_2$ complex. This hypothesis is supported by the comparison of fluorescence spectra of 1 recorded in both salt-free and salt-rich environments (Figure S10B), an indication that chloride has no measurable fluorescence-quenching effect on the hydrolyzed product (i.e., fluorescein) at these concentration levels.

Fluorescence Response of 2 Toward Hydrolysis. To investigate the optical properties, hydrolytic behavior, and sensing ability of intermediary compound 2 identified through mass spectrometry (MS) analysis, the latter was synthesized and isolated by modifying the original synthesis protocol to favor a single-fold condensation instead of a twofold reaction (Scheme 6). In a similar fashion to the free fluorescein acid, the fluorescein monoether derivative may exist as a tautomeric equilibrium between its lactone and quinoid structures. However, in opposition to compound 1, the $^{13}$C NMR of 2 does not indicate a signal attributed to the sp$^3$-hybridized carbon atom of a spirolactone (Figure S4), suggesting that the molecule exists as the ring-opened quinoid xanthene in CDCl$_3$. Moreover, UV–visible and fluorescence spectra in water-free chloroform have confirmed that 2 does neither absorb significantly in the visible region nor exhibit fluorescence when excited at 490 nm (results not shown). These findings suggest that even though the molecule is present in its quinoid form, it is nonemissive. This is due to the inhibition of ICT that is otherwise occurring between the electron-donating phenol and electron-withdrawing carbonyl moieties appended to the xanthene core of fluorescein. In compound 2, the electron-donating ability of the phenol is restricted by its bonding to the thionocarbonate moiety. Therefore, isolation of the ICT-inhibited compound 2 supports the hypothesis that fluorescein is the only emitting species resulting from the hydrolytic processes under study with compound 1.

To compare the hydrolytic reactivity of compounds 1 and 2, the emission spectrum of as-synthesized 2 was measured over time in a HEPES buffer solution (20 mM, pH 7.4, 1% EtOH) (Figure 7A). The spectrum of compound 2 alone reveals an emission band characteristic of free fluorescein when excited at fluorescein’s absorption maximum ($\lambda_{\text{exc}} = 490$ nm). Since the pure quinoid molecule has been determined to be nonemissive in water-free solvents, this signal is hypothesized to be caused by the autohydrolysis of the carbonothioate moiety. Indeed, similarly to experiments with probe 1, the autohydrolysis of 2 leads to an increasing fluorescence band at 516 nm, reaching a 12-fold fluorescence intensity after 90 min ($I_{90}/I_0$). The probe’s fluorescence increases more rapidly than probe 1, with a rate $r_2$ of 645 s$^{-1}$ compared to 74 s$^{-1}$ for $r_1$ in the same conditions (Table 1). This faster pace is most likely explained by a single-fold hydrolysis process compared to the dicarbonothioated version, which must undergo two hydrolysis steps per molecule to yield the emissive form. Moreover, the study of this intermediate confirms that its hydrolysis is not a limiting step in the two-step consecutive hydrolysis of probe 1 proposed in Scheme 4.

Figure 6. (A) Dependence to pH of normalized reaction kinetics $r$ of 1. (B, C) Excitation (dotted) and emission (full) spectra of probe 1 in the presence of 2 equiv of Hg$^{2+}$ recorded between pH 3–7 and 7–10, respectively. Conditions: 1 $\mu$M of 1 in a HEPES solution (20 mM, 1% EtOH) at 25 °C. All excitation spectra taken at $\lambda_{\text{em}} = 516$ nm. Emission spectra taken at the respective maximum $\lambda_{\text{exc}}$ previously acquired. Error bars represent RSD ($n = 3$).

Scheme 6. Synthesis of 2 and Its Tautomerization Reaction
The sensitivity of compound 1 toward Hg$^{2+}$ was examined between 0 and 2 equiv of Hg$^{2+}$ (0–2 μM) (Figure 8). The linear range extends to 1.6 μM, at which point the kinetic rate plateaus. The detection limit for Hg$^{2+}$ using probe 1 was calculated as 0.8 nM (3σ/slope), with the signal background due to spontaneous hydrolysis being the limiting factor. The resulting linear range extends over more than 3 orders of magnitude. The tolerable level of mercury contamination in drinking water being set at 1 ppb (5 nM) by WHO, this result demonstrates that a 1 μM solution of compound 1 has the potential to detect Hg$^{2+}$ at relevant concentrations. Hence, this study is an important addition to the known library of thionocarbonated dyes as it demonstrates competitive performance, but mostly a better comprehension of limitations of these hydrolysis-based Hg probes.

A calibration curve was also determined for intermediate compound 2 to compare the sensitivity of 1 and 2 toward Hg$^{2+}$ sensing (Figure S11). The result shows a linear range reaching 0.6 equiv of Hg$^{2+}$. The narrower linearity range of 2 can be explained by the presence of only one reactive site per molecule. The detection limit of probe 2 was calculated as 40 nM (3σ/slope). Although the faster hydrolysis of compound 2 leads to a steeper slope than that obtained for 1, the high spontaneous hydrolysis severely impacts the LOD achieved.

### CONCLUSIONS

To summarize, an Hg$^{2+}$-responsive thionocarbonate-appended fluorescent probe was developed using a one-step synthesis. The contribution of auto- and Hg$^{2+}$-assisted hydrolyses to the probe’s overall operation was investigated by kinetic fluorescence monitoring and mass spectrometry. The role of Hg$^{2+}$ as a soft Lewis acid catalyst accelerating the consecutive hydrolytic processes was supported by a selectivity assay, a temperature-based experiment, and the isolation of an intermediate compound. The influence of pH and chloride ions on the probe’s response to Hg$^{2+}$ was also characterized, stressing the importance of speciation on the availability of Hg$^{2+}$ for sensing. Overall, this addition to the library of available thionocarbonated dyes and the insights into their sensing mechanism will be instrumental in designing reliable and effective portable sensing strategies for mercury and other thiophilic metals.

### EXPERIMENTAL SECTION

#### Chemicals and Materials

Fluorescein acid, o-phenyl chlorothionoformate, o-phenyl chloroformate, diisopropylthylamine (DIPEA, 99.5%), HEPES (99.5%), and NaCl were purchased from Sigma-Aldrich. Dry dichloromethane was purchased from ACROS Organics. Anhydrous ethanol was purchased from GreenField Global. Ethylacetate and hexanes were purchased from Fisher Scientific. Sodium hydroxide was purchased from J.T. Baker. Hg$^{2+}$, Zn$^{2+}$, Na$^+$, Pb$^{2+}$, Ag, Co$^{2+}$, Cu$^{2+}$, K$^+$, Ni$^{2+}$, Al$^{3+}$, Cd$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Mg$^{2+}$, Ba$^{2+}$, Cs$^{2+}$, Sr$^{2+}$, Li$^+$, and Cr$^{3+}$ solutions were prepared from standard solutions purchased from SPC Science. All chemicals were used without further purification. Flash column chromatography was performed on a 230–400 mesh silica gel R10030B purchased from Silicycle (Canada).

#### Instrumentation

Nuclear magnetic resonance (NMR) spectra were recorded with a Varian Inova AS400 spectrometer (Palo Alto) at 400 MHz (1H) and 100 MHz (13C). High-resolution mass spectra (HRMS) were recorded with an Agilent 6210 time-of-flight (TOF) LC–MS apparatus equipped with an ESI ion source (Agilent Technologies, Canada). UV–visible absorption spectroscopy was performed.
with a Varian Cary 50 spectrophotometer using 10 mm path length quartz cells. Fluorescence spectra were acquired on a Fluorolog 3 spectrofluorimeter (Jobin-Yvon Horiba).

Synthesis of Compound 1. In a round-bottom flask equipped with a stir bar, fluorescein acid (0.300 g, 0.91 mmol) was dissolved in degassed dry dichloromethane (6.5 mL). Diisopropylethylamine (316 μL, 1.81 mmol) and o-phenyl chlorothionoformate (377 μL, 2.33 mmol) were added sequentially, and the reaction vessel was stirred at room temperature for 16 h. The solvent was removed under reduced pressure, and the crude material was purified by column chromatography (silica gel, 25:75 ethylacetate/hexanes as eluent) to yield 0.219 g (63%) of compound 1 as a beige solid.1H NMR (400 MHz, DMSO- d6) δ 8.04 (dt, J = 7.4, 1.0 Hz, 1H), 7.80 (td, J = 7.5, 1.3 Hz, 1H), 7.74 (td, J = 7.5, 1.1 Hz, 1H), 7.54 (d, J = 2.5 Hz, 1H), 7.52–7.43 (m, 4H), 7.41 (dt, J = 7.6, 1.0 Hz, 1H), 7.37–7.24 (m, 6H), 7.13 (dd, J = 8.7, 2.4 Hz, 2H), 6.98 (d, J = 9.1 Hz, 2H). 13C NMR (400 MHz, DMSO- d6) δ 194.0, 168.7, 154.7, 153.6, 152.4, 151.3, 136.6, 131.1, 130.4, 130.1, 127.5, 125.8, 125.6, 124.6, 122.1, 119.3, 117.8, 115.7, 111.4, 81.1. HRMS (ESI): m/z calcld for C34H20O7S2+H+: 605.0723 [M + H]+; found, 605.0706.

Synthesis of Compound 2. In a round-bottom flask equipped with a stir bar, fluorescein acid (0.300 g, 0.91 mmol) was dissolved in degassed dry dichloromethane (180 mL). Diisopropylethylamine (106 μL, 0.61 mmol) and o-phenyl chlorothionoformate (126 μL, 0.91 mmol) were added sequentially, and the reaction vessel was stirred at room temperature for 16 h. The solvent was removed under reduced pressure, and the crude material was purified by column chromatography (silica gel, 50:50 ethylacetate/hexanes as eluent) to provide 0.035 g (8%) of compound 2 as an orange solid.1H NMR (400 MHz, CDCl3) δ 8.8, 1.4 Hz, 1H), 7.70 (td, J = 7.6, 1.2 Hz, 1H), 7.64 (td, J = 7.4, 1.2 Hz, 1H), 7.46 (td, J = 7.6, 2.2 Hz, 2H), 7.34 (td, J = 7.4, 1.2 Hz, 1H), 7.19–7.24 (m, 3H), 7.18 (d, J = 2.2 Hz, 1H), 6.92 (dd, J = 8.8, 2.2 Hz, 1H), 6.86 (d, J = 8.6 Hz, 1H), 6.77 (d, J = 2.2 Hz, 1H), 6.61 (d, J = 8.6 Hz, 1H), 6.56 (dd, J = 8.2, 2.4 Hz, 1H), 13C NMR (400 MHz, CDCl3) δ 193.9, 171.6, 169.9, 159.6, 156.9, 154.6, 153.4, 152.8, 152.2, 152.1, 135.4, 130.0, 129.7, 129.3, 129.2, 127.0, 126.4, 121.5, 117.9, 117.6, 117.2, 112.8, 110.9, 110.6, 107.8, 103.2. HRMS (ESI): m/z calcld for C27H16O6S+H+: 469.0720 [M + H]+; found, 469.0811.

Synthesis of Compound 3. In a round-bottom flask equipped with a stir bar, fluorescein acid (0.200 g, 0.60 mmol) was dissolved in degassed dry dichloromethane (6 mL). Triethylamine (253 μL, 1.80 mmol) and o-phenyl chlorothionoformate (182 μL, 1.44 mmol) were added sequentially, and the reaction vessel was stirred at room temperature for 2 h. Dichloromethane (20 mL) and an aqueous phase were neutralized with NaOH. The organic phases were combined and dried over anhydrous Na2SO4. The crude material was purified by column chromatography (silica gel, 35:65 ethylacetate/hexanes as eluent) to yield 0.219 g (63%) of compound 3 as a white solid.1H NMR (400 MHz, CDCl3) δ 8.06 (d, J = 7.4, 1.1 Hz, 7.71 (td, J = 7.4, 1.4 Hz, 1H), 7.66 (td, J = 7.4, 1.4 Hz, 1H), 7.43 (t, J = 7.8 Hz, 1H), 7.32–7.26 (m, 8H), 7.20 (dt, J = 7.4, 1.0 Hz, 1H), 7.03 (d, J = 2.4 Hz, 1H), 7.01 (d, J = 2.4 Hz, 1H), 6.89 (d, J = 8.5 Hz, 1H), 13C NMR (400 MHz, CDCl3) δ = 194.5, 169.0, 152.8, 152.2, 151.5, 150.8, 135.4, 130.2, 129.7, 129.2, 126.5, 126.0, 125.4, 124.0, 120.8, 117.1, 117.0, 110.9, 81.3. HRMS (ESI): m/z calcld for C27H36O2H+: 573.1180 [M + H]+; found, 573.1261.

Stock Solutions. The 20 mM HEPES buffer solution was prepared from the corresponding commercial salt dissolved in water, and its pH was adjusted with HNO3 and NaOH solutions using a pH meter. Stock solutions (100 μM) of compounds 1–3 were prepared by dissolving the solids in anhydrous EtOH. To prevent spontaneous hydrolysis during storage, the probes were always kept as solids or in water-free ethanol stock solutions preserved at 4 °C for up to 2 weeks without significant hydrolysis. A 100 μM Hg2+ stock solution was prepared by diluting and neutralizing a 5 mM ICP standard solution with NaOH in nanopure water. All other cation solutions were prepared in a similar fashion from their neutralized commercially available ICP standard solutions.

Instrumental Considerations for Kinetic Fluorescence Monitoring. For all kinetic-based fluorescence results, the spectrophotometer was set with the following parameters: 1 or 2 nm slits, λexc = 490 nm, λem = 516 nm, Δt = 15 s with the shutter closed between data points to minimize photobleaching. All samples were continuously stirred with a magnetic stir bar in the quartz cell, and the temperature was equilibrated 2 min before starting all kinetic studies. Each sample was measured in two or three replicates, and the treated data was averaged with the corresponding standard deviation taken as the uncertainty. All final data for a given experiment were then normalized to prevent instrumental fluctuations from affecting data interpretation across different experiments. Absolute values were only used for the calibration curves.

Sample Preparation for Kinetic Fluorescence Monitoring. To 1.98 mL of HEPES buffer in a quartz cell was added 20 μL of I stock solution. The cuvette was placed in the spectrometer’s sample compartment to acquire autohydrolysis data. Fluorescence measurements were taken at intervals of 15 s over a total time of 120 s. For Hg2+-assisted hydrolysis, the probe-containing cuvette was spacked with 40 μL of the analyte stock solution, and fluorescence measurements were again taken each 15 s over a total time of 780 s. For both situations, the data was plotted as intensity (I) vs time (t) and the derivative was taken to report rprobe and rprobe+Hg2+. For selectivity experiments, the cuvette and buffer solutions were left to equilibrate at the desired temperature before adding compound 1.

For selectivity studies, the 100 μM stock solution of Hg2+ was replaced with 100 μM solutions of selected cations. For sensitivity experiments, Hg2+ stock solutions of 0–100 μM were prepared to preserve the volume (40 μL) spiked in the cuvette across all standards.

Sample Preparation for Mass Spectrometry. Of a 500 μM stock solution of 1 in EtOH, 90 μL was added to 0.9 mL of either pure water or a 100 μM Hg2+ stock solution. The EtOH content was adjusted to 10% v/v by adding 10 μL of the latter. The sample was placed in the apparatus, and data was acquired 5 min after solution preparation. One can note that the concentrations used are 45 times higher than those for fluorescence studies to yield proper MS detection. The use of a larger proportion of the EtOH-based stock solution of 1 implies higher EtOH content in the sample than for fluorescence studies. The impact of this EtOH content on hydrolysis has been swiftly investigated and was found to slightly decrease the fluorescence kinetics, r, either from a slower reaction or from a less efficient emission of the generated fluorescein.

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1H and 13C NMR spectra of compounds 1–3; time-dependent fluorescence spectra of compound 3; reaction kinetics of 1 in the presence of Hg2+ and other ions; fluorescence spectra of 1’s autohydrolysis according to pH; effect of NaCl concentration on reaction kinetics of 1; reaction kinetics of 2 as a function of Hg2+ concentration; and prototropic and tautomeric forms of fluorescein (PDF)

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