Synthesis and characterization of a novel fluorescent and colorimetric probe for the detection of mercury (II) even in the presence of relevant biothiols

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ARTICLE INFO

ABSTRACT

We present here a novel probe, coumarin 3-azomethine derivative (**AGB**), which can detect selectively mercuric ions (Hg^{2+}) via a hydrolysis reaction promoted by these ions. Interestingly, the probe can be used to assess the response to Hg^{2+} even in the presence of the most important biothiols-glutathione and cysteine—and could be used in the Hg^{2+}—imaging in living cells.

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Mercury is a heavy transition metal that generally possesses three distinct oxidation states 0, +1, and +2, although quantum chemical predictions have also suggested the existence of mercury in its oxidation state +4.[1] Among these, the mercuric state Hg^{2+}, is considered one of the most toxic and dangerous forms due to its very high affinity for sulfhydryl groups. In this context, the interaction between mercuric ions and glutathione (GSH) leads to favorable generation of different mercury containing GSH-complexes. For example in the case of the Hg^{2+}–$[GSH]_2$ complex[2–4] a high affinity between Hg(II) and GSH, with stability constant ($\log \beta \geqslant 20$), has been reported.[3] Additionally, considering that the intracellular concentration of GSH is high (0.5–10 mM)[5] the presence of free mercury ions, in biologically relevant conditions, would be very low and thus the metal would be predominantly involved in sulfur-containing complexes. In consequence, the latter would limit the detection of free mercuric ions in such conditions.

Different probes based on rhodamine,[6–8] coumarin,[9] and squaraine derivatives,[10] as well as other chromophores,[11–13] have been developed to detect the presence of free (or labile) mercury (II) ions. Nonetheless, currently few reports discuss how to avoid the

potential interference from sulfur-rich environments (namely in the presence of GSH and cysteine) in the detection of Hg^{2+}.[12,13a]

On the other hand, it is known that chromophores containing azomethine groups (also called Schiff bases) have been used as sensors of metals, mainly by taking advantage of the suppression of C=N isomerization due to the binding with the metal.[14,15] In this context, several studies[14,15] have indicated that as a consequence of this binding a restriction of such isomerization occurs and a significant fluorescence enhancement is observed.

Nonetheless, probes containing the azomethine group can also act as chemodosimeters via hydrolysis reaction promoted by metals.[13,16,17] In this line, only a few probes have been developed to selectively detect Hg^{2+} ions via the hydrolytic pathway of the azomethine group.[13]

Prompted by the latter, we present here a novel probe based on a coumarin-3-azomethine derivative linked to O-(4-formylphenyl) dimethylcarbamothioate, which could be useful for Hg^{2+} detection even in the presence of biologically relevant thiols.

The new probe, (E)-O-(4-(((7-hydroxy-2-oxo-2H-chromen-3-yl) imino)methine)phenyl)dimethylcarbamothioate (**AGB**) was prepared via a conventional four-step synthesis from commercial precursors. The first step involves a Vilsmeier–Haack formylation of resorcinol to obtain 2,4-dihydroxybenzaldehyde, which is subsequently condensed with N-acetylglycine (by a Knoevenagel
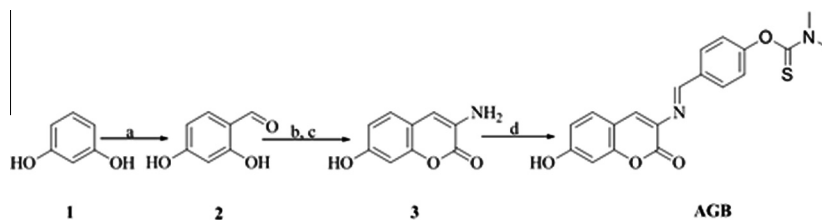
Scheme 1. Synthetic route to **AGB**. Reagents and conditions: (a) POCl$_3$, DMF, acetonitrile, 0–5 °C, 2 h; (b) acetylglycine, acetic anhydride, anhydrous sodium acetate, reflux 4 h; (c) 2:1 HCl/H$_2$O reflux, 2 h; (d) O-(4-formylphenyl) dimethylcarbamothioate, EtOH, reflux, 4 h.
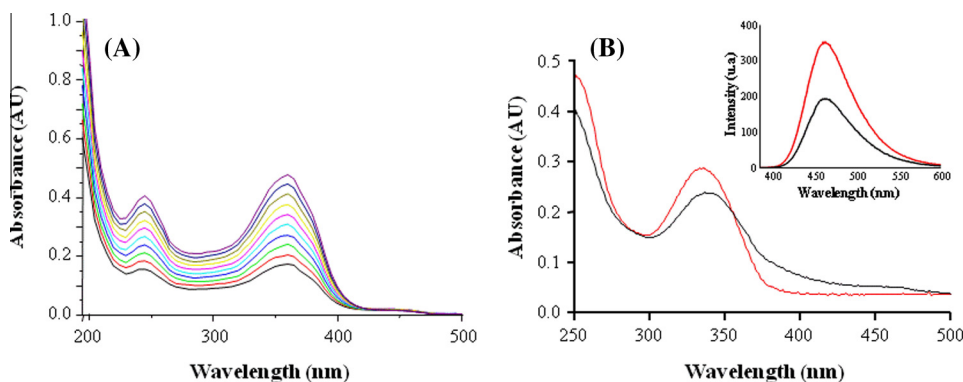
Figure 1. (A) Absorption spectra of **AGB** (5–100 μM) in a DMSO/H$_2$O (1:99, v/v). (B) Absorption spectra of **AGB** (20 μM) solution in the absence (black line) or in the presence of 10 equiv Hg^{2+} (red line), after 5 min of incubation at 25 °C; insert represents the emission spectra of **AGB** (5 μM) in a DMSO/H$_2$O (1:99, v/v) solution in the absence (black line) or in the presence of 10 equiv Hg^{2+} (red line).

reaction) and hydrolyzed in situ to afford 3-amino-7-hydroxy-coumarin. The final step is the condensation of 3-amino-7-hydroxycoumarin with O-(4-formylphenyl) dimethylcarbamothioate to yield the final product **AGB** (Scheme 1), which was characterized by ^{1}H NMR, ^{13}C NMR spectroscopy (Figs. S1 & S2, Supporting information), and by high-resolution mass spectrometry (HRMS) (Fig. S3, Supporting information).

Once the structure of the compound **AGB** was confirmed, some photophysical properties of **AGB** were assessed. Regarding the absorption spectrum of **AGB** it depicts two bands with maxima at ~240 nm and ~350 nm (Fig. 1A). These bands might be mainly associated with π → π* transitions and an overlap of π → π* with n → π* transitions, according to what is reported for similar compounds.[18,19] The maximum of the absorption band of **AGB** at 350 nm exhibits an extinction coefficient (ε) of 16021 M^{-1} cm^{-1} (Fig. S4, Supporting information).

When excited at 350 nm, the emission spectrum of **AGB** exhibits a maximum at 460 nm, as shown in insert to Figure 1B, (in black color). However, the emission intensity associated with the compound **AGB** is low (quantum yield of 0.01), probably attributed to a decay process of the C=N bond cis–trans isomerization, as mentioned above.[14]

Upon the addition of mercuric ions to a solution containing **AGB**, we found that the absorption band at 350 nm undergoes a small hypsochromic shift (~15 nm) and at least two zones of the spectrum suffer spectroscopic changes, as shown in Figure 1(B). On the other hand, when excited at 350 nm, a fluorescence intensity enhancement was observed at 460 nm (insert Fig. 1B). These changes in the UV–vis and emission spectra could be associated with the formation of a new species after the interaction between mercuric ions and **AGB**.

To ensure that **AGB** only interacts with Hg^{2+}, we examined the effects of various metal ions on the spectral features of **AGB**. Variation of the absorption maximum spectra of **AGB** upon the addition of different metal cations including Li$^+$, Na$^+$, K$^+$, Mg^{2+}, Ca^{2+}, Ba^{2+}, Al^{3+}, Cu^{2+}, Cd^{2+}, Fe^{3+}, Ag$^+$, Mn^{2+}, Pb^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+} is shown in

Figure 2. The absorption maximum shows no obvious changes upon the addition of such tested metals. In addition, selectivity experiments were also carried out using fluorescence spectroscopy and we found no interferences of other ions on the response of **AGB** (not shown).

Once the selectivity of our probe was assessed, we determine the limit of detection (LOD) of **AGB** toward mercuric ions. With this aim, we carried out titration experiments of **AGB** by the incremental addition of Hg(II) ions (from 20 to 200 μM). The plot in Figure 3 shows a linear behavior (r^2 = 0.987, k = −726.2 au M^{-1}) with a standard deviation of σ = 0.0054. The LOD calculated (3σ/k) gave a result of 2.2 × 10^{-5} M (as colorimetric probe) and 4.5 × 10^{-6} M (as fluorescent probe). These values suggest the use of **AGB** as colorimetric or fluorescent probes for Hg^{2+} either in environmental and/or biological applications.
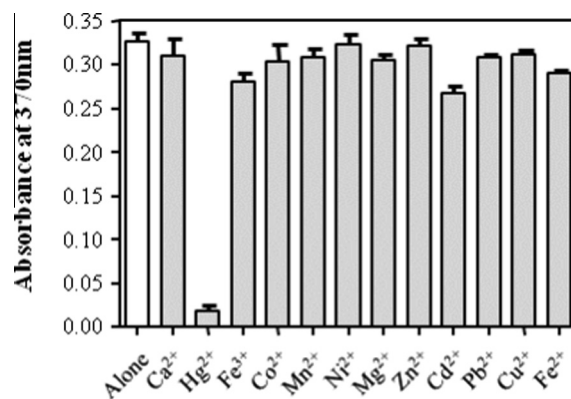
Figure 2. Selectivity of the **AGB** probe (15 μM) toward Hg^{2+} ions (200 μM) in the presence of other metal ions: Hg^{2+}, Fe^{3+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Ni^{2+}, Pb^{2+} or Cd^{2+} (200 μM). The white bar represents the **AGB** probe alone and the light gray bars represent solutions containing **AGB** plus different metal ions, after 5 min of incubation at 25 °C.
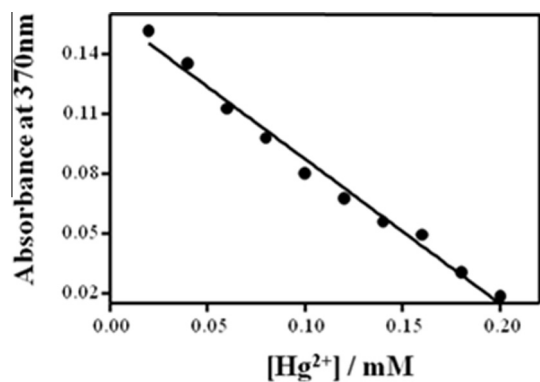
Figure 3. Plot of absorbance at 370 nm of **AGB** (20 μM) against concentrations of Hg²⁺ from 20 to 200 μM, in aqueous solution.

Figure 5. Plot of k_{obsd} vs concentration of Hg²⁺ ions for the interaction of **AGB** with Hg²⁺ at different pHs values.

Aiming to explore the mechanism of mercury-induced signal suppression of **AGB** (Fig. 3), we performed kinetic studies to evaluate the response of this coumarin-3-azomethine derivative to Hg²⁺, under pseudo-first-order reaction conditions, at 25 °C. The time-dependent response of **AGB** to mercury ions was monitored by UV–vis spectroscopy.

As previously mentioned and as shown in Figure 4(A), after the addition of mercuric ions to a solution containing **AGB**, a new absorption band (near 340 nm) appears as a consequence of the decrease of the band at 370 nm. This process was completely finished after 100 s of reaction at pH = 6.0 (Fig. 4(B)). In this period of time, probably a decomposition reaction of **AGB** promoted by Hg²⁺ is occurring.

It is important to note that the kinetic profiles clearly indicate the fast response of our probe to Hg²⁺ in comparison with other mercury probes.[12,13a]

In this context, Figure 5 shows the variation of the pseudo-first-order rate constants (k_{obsd}) for the proposed decomposition reaction of **AGB** with pH values (between 5.0 and 7.0, data from Table S1).

We found that the reaction rate strongly increases at low pH values, whereas at pH >8 no significant reaction was observed (data not shown). The latter behavior permits to propose an acid-catalytic process as the rate-determining step in the decomposition of coumarin-3-azomethine derivative induced by mercuric ions. Under our experimental conditions, the reaction stoichiometry between **AGB** and Hg²⁺ ions was determined to be 2:1 from the Job's analysis[9b,11d] (Fig. S5, Supporting information). This result also suggests a catalytic participation of mercury in the process.

To gain information on the kind of reaction that **AGB** undergoes in the presence of Hg²⁺, NMR and HRMS analyses were carried out.
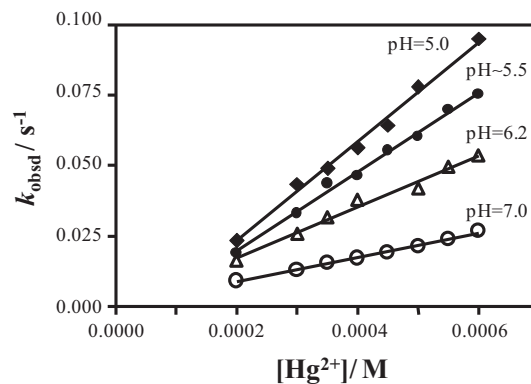
Figure 6 presents the spectra of **AGB** (20 mM in part A) and that resulting from mixing such a compound with three equivalents of Hg²⁺ ions (part B). This figure shows that once these ions are added, the Hb proton signal of **AGB** at 7.8 ppm (Fig. 6(A)) shifts upfield to 6.8 ppm (Fig. 6(B)), accompanied by more subtle changes in the rest of the aromatic region of the spectrum.

Concerning the signal of the imine proton Ha, at δ = 8.95 ppm, it disappears and a typical aldehyde signal arises at δ = 9.95 ppm. Therefore, the NMR spectrum presented in Figure 6(B) could be associated with the presence of an amino-hydroxycoumarin and a benzaldehyde derivative. In consequence we suggest that the decomposition of **AGB** corresponds to a hydrolysis reaction where the principal products would be O-(4-formylphenyl) dimethylcarbamothioate and 3-amino-7-hydroxycoumarin (see the structure in spectrum B).

With the aim to demonstrate the importance of the presence of mercuric ions in the studied reaction, a control experiment was carried out in the absence of these ions. In this case the ¹H NMR of **AGB** remained unchanged at least during the time that its reaction with Hg²⁺ was ended.

Regarding ¹³C NMR experiments, we observed that upon addition of Hg²⁺ ions to a solution of **AGB**, the signal at 164 ppm disappears while a new peak at 193 ppm is formed. The former may be associated with the corresponding carbon of azomethine fragment while the latter may correspond to the aldehyde carbon present in the O-(4-formylphenyl) dimethylcarbamothioate (Fig. 7). This result corroborates the proposed hydrolysis reaction of **AGB** promoted by Hg²⁺.

On the other hand, it is well known that Hg²⁺ ions are able to induce a desulfurization reaction on a sulfur atom linked to carbon
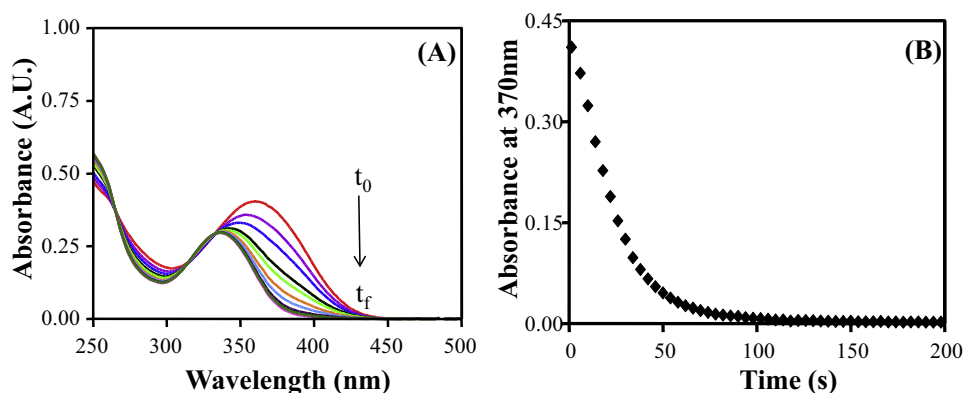
Figure 4. (A) Time dependent UV–vis spectra of **AGB** (20 μM) upon addition of HgCl₂ (200 μM) in 20 mM PBS buffer, pH 6.0; t_0 and t_f represent initial and final time (100 s), respectively. (B) Kinetic profile for the interaction between **AGB** (20 μM) and Hg²⁺ (600 μM), at 25 °C.
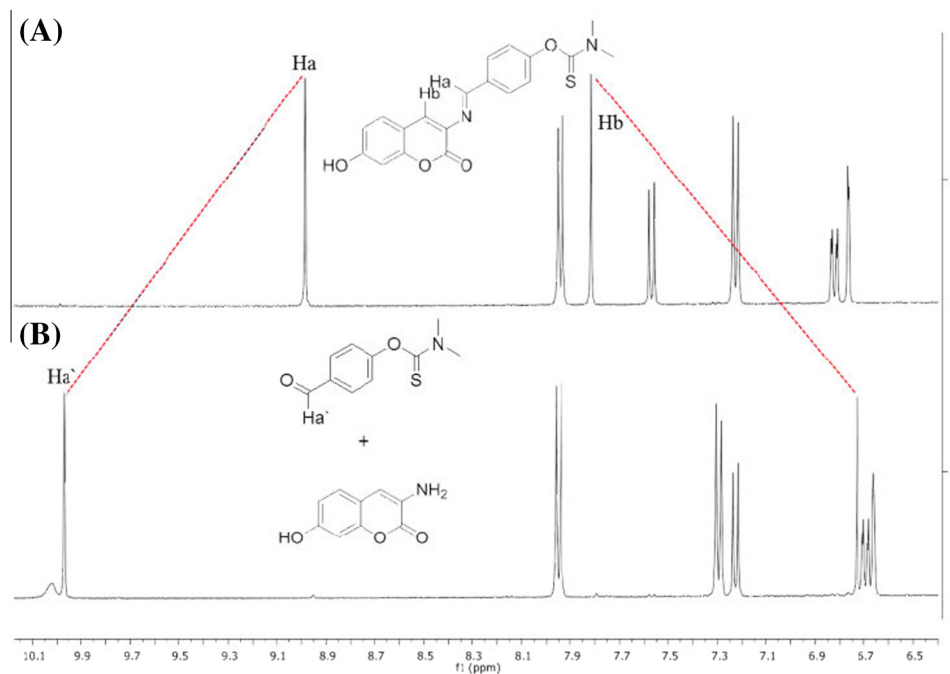
Figure 6. ^{1}H NMR spectra of **AGB** in DMSO-d_6 in the absence (A) and in the presence (B) of 3 equiv of HgCl$_2$ in DMSO-d_6/D$_2$O 9:1.
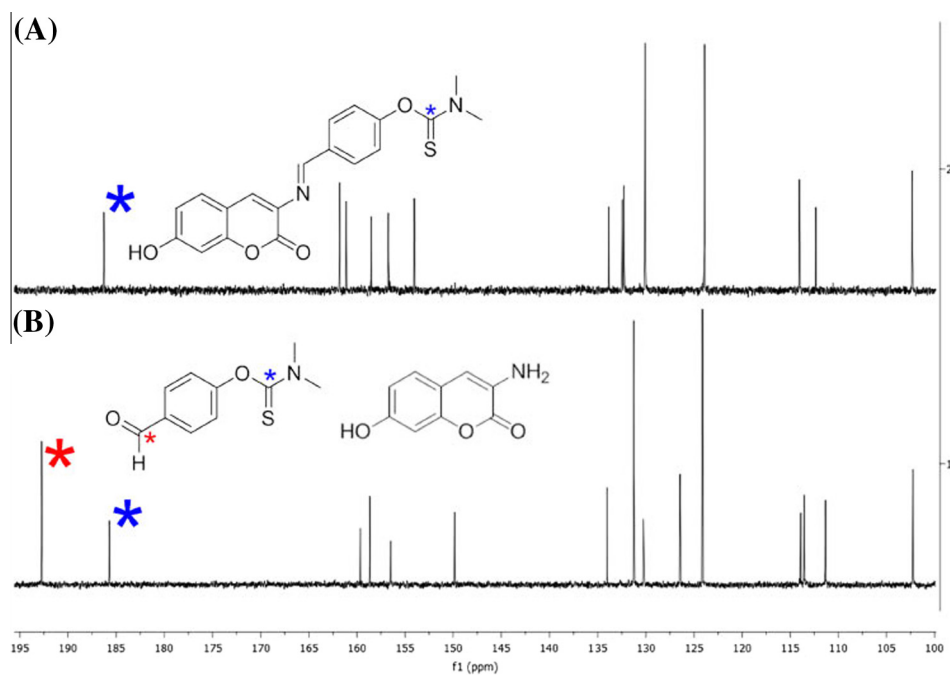

Figure 7. ^{13}C NMR spectra of **AGB** in DMSO-d_6 in the absence (A) and presence (B) of 3 equiv of HgCl$_2$ in DMSO-d_6/D$_2$O 9:1.

(C=S).[8] However, this reaction was discarded, at least in our experimental conditions, considering the results obtained from ^{13}C NMR (Fig. 7). These results show that the observed signal at δ = 186.3 ppm, attributable to C=S present in **AGB**, was not modified after the addition of mercuric ions.

The presence of both proposed products of reaction, O-(4-formylphenyl) dimethylcarbamothioate and 3-amino-7-hydroxycoumarin, is also supported by HRMS (in positive mode) studies

(Fig. S6, Supporting information). Molecular ion peaks at m/z 209.0387 [M]$^+$ and 179.0167 [M+2H)]$^+$, respectively, in the HRMS-ESI spectra of **AGB** in the presence of Hg^{2+} strongly support their formulations.

Considering these results, in particular the formation of 3-amino-7-hydroxycoumarin, it is easy to explain the hypsochromic shift observed for the absorption band of **AGB** from ~360 nm to 340 nm when Hg^{2+} is added (Fig. 1B). This shift would be a

Scheme 2. Proposed mechanism of Hg^{2+}-induced imine hydrolysis of **AGB**.
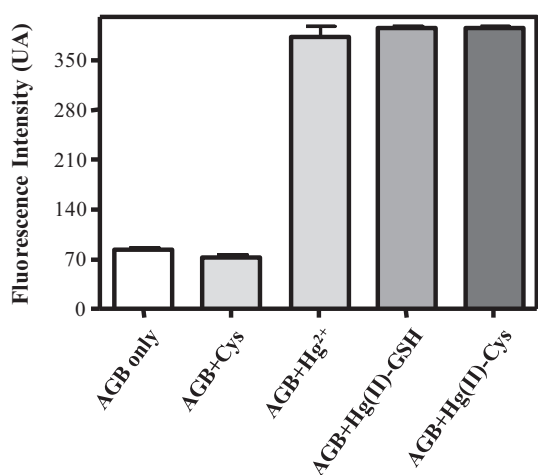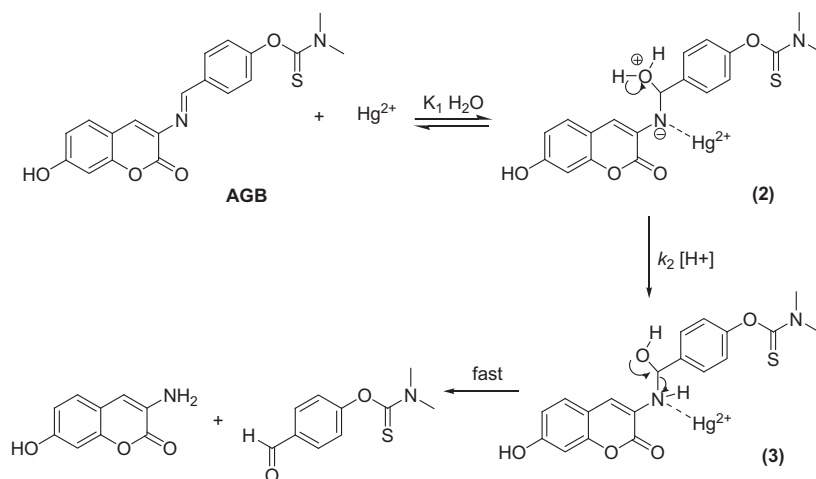
Figure 8. Detection of Hg^{2+} (200 µM) in the presence of glutathione or cysteine (500 µM) in HEPES buffer (pH 7.0, 20 mM) by **AGB** (10 µM). $\lambda_{exc.}$ = 340 nm and $\lambda_{em.}$ = 460 nm.

consequence of the absorption properties of 3-amino-7-hydroxy-coumarin, namely an absorption spectrum centered at 340 nm, which has been recently reported by us.[20]

It is important to note that, when mercuric ions are added into a solution containing 3-amino-7-hydroxycoumarin, no changes are observed in the UV–vis spectra between 300 and 450 nm at least during 1 h after mixing (not shown). Thus, the possibility that this product reacts with Hg^{2+} is discarded.

Additionally, taking into account the results of kinetic studies and product analysis the most probable mechanism is that described in Scheme 2, where the first step involves the reaction of water with **AGB**, which is promoted by mercuric ions. Then in a second step the intermediate (2) is protonated by the reaction media and finally occurs as the fast decomposition of hemiaminal intermediate (3) of **AGB** occurs to yield O-(4-formylphenyl) dimethylcarbamothioate and 3-amino-7-hydroxycoumarin.

In view that some important sulfhydril containing biomolecules, such as the aminoacid cysteine (Cys) and the tripeptide glutathione (GSH),[4] could interact with Hg^{2+}, we investigated the effect of these endogenous thiols on the detection of Hg^{2+} in neutral conditions (HEPES buffer solutions pH 7.0, 20 mM). As shown in Figure 8, the addition of **AGB** to a solution containing Cys (500 µM) does not induce a change in the fluorescence response in comparison with **AGB** alone. However, when **AGB** was added to a solution of Hg^{2+} (200 µM) in the presence of Cys a considerable increase of the fluorescence intensity was observed, which is very close to the increment induced only by Hg^{2+}.

A similar result was obtained when GSH was assessed instead of Cys and when the absorbance at 370 nm was taken into account (Fig. S7, Supporting information). This suggests that **AGB** could detect Hg^{2+} in the sulfur rich environment.

Finally, and taking advantage of the observed changes in the fluorescence response of **AGB** in the presence of mercury ions, free and bound to biothiols, we assessed the **AGB** probe for in vitro detection of Hg^{2+} ions in SH-SY5Y neuroblastoma cells. The cell lines were incubated with **AGB** (5.0 µM) in culture medium for 30 min at 37 °C; a weak fluorescence of **AGB** inside the living cells was observed (Fig. 9A). However, when the living cells were
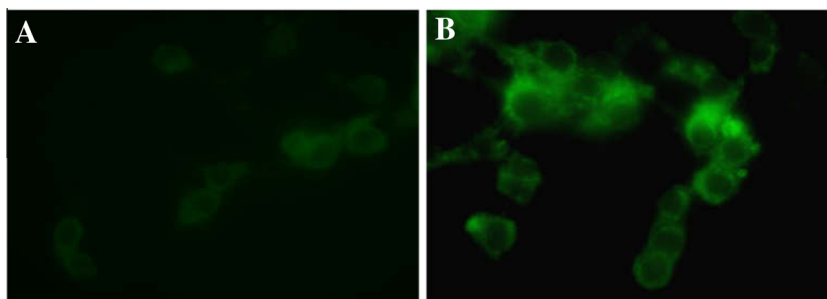
Figure 9. Fluorescence images of SH-SY5Y cells lines. (A) Fluorescence images of cells incubated with 5 µM of the **AGB** probe. (B) Fluorescence images of cells upon treatment with **AGB** (5 µM) and then with $HgCl_2$ (10 µM) for 30 min.

incubated with Hg^{2+} (10 μM) in the medium for another 30 min at 37 °C, the fluorescence in cells was much higher (Fig. 9B).

The increase in the fluorescence observed in Figure 9 would be a consequence of the generation of 3-amino-7-hydroxycoumarin by the Hg^{2+}-mediated hydrolysis of **AGB**. Therefore, considering that the **AGB** probe is cell permeable and taking into account the above results, we proposed that **AGB** is useful as an intracellular Hg^{2+} ion imaging probe.

In conclusion, a 3-azomethine derivative of coumarin dye (**AGB**) was synthesized as a selective fluorescent and colorimetric probe for Hg^{2+} in aqueous media. Interestingly, this probe is able to detect mercuric ions even in the presence of cysteine or glutathione via an acid-catalyzed hydrolysis process. Most importantly, this probe was successfully applied to the imaging of Hg^{2+} ions in cells.

Acknowledgments

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2015.09.001.

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