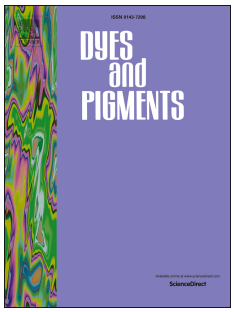

Synthesis, spectroscopic and computational evaluation of a xanthene-based fluorogenic derivatization reagent for the determination of primary amines

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Dyes and Pigments

Research Article

CRediT authorship contribution statement

— **Amalia D. Kalampaliki:** Methodology, Formal analysis, Investigation. **Steve Vincent:** Methodology, Formal analysis, Investigation. **Suman Mallick:** Methodology, Investigation, Review. **Hoang-Ngoan Le:** Visualization, Project administration. **Guillaume Barnoin:** Visualization, Validation. **Yogesh W. MORE:** Validation, Data curation. **Alain BURGER:** Funding acquisition, Review. **Yannis Dotsikas:** Conceptualization, Investigation. **Evagelos Gikas:** Funding acquisition, Writing – review & editing. **Benoît Y. MICHEL:** Funding acquisition, Writing – review & editing, Supervision. **Ioannis K. Kostakis:** Funding acquisition, Writing – review & editing, Supervision.

Graphical Abstract

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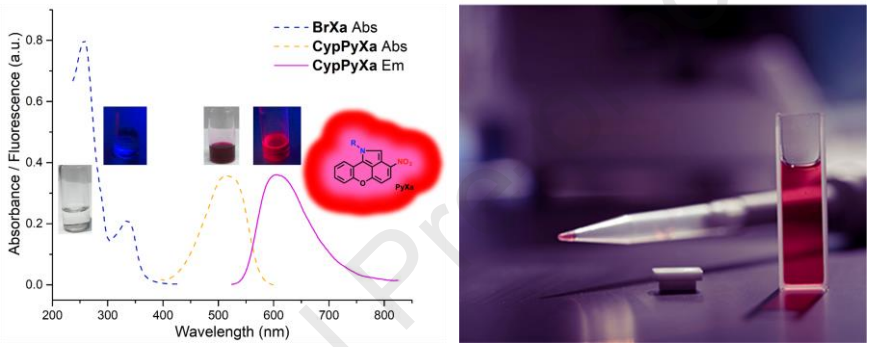

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Synthesis, spectroscopic and computational evaluation of a xanthene-based fluorogenic derivatization reagent for the determination of primary amines

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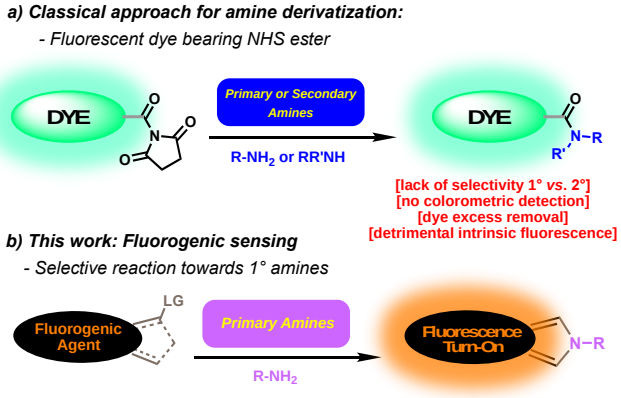
ABSTRACT

To detect an analyte, typically at the sub-nanomolar scale, extremely sensitive analytical tools are required. Fluorescence is the spectroscopy of choice to achieve such a level due to its non-invasive nature and efficiency in accurately probing the sub-nanomolar concentration range. Here, we report the design, synthesis and photophysical characterization of a fluorogenic derivatization reagent with exclusive selectivity for primary amines. This xanthene-based dye owns an exacerbated fluorogenic character making the derivatized amine absorbing in yellow and emitting in the vermilion edge. In addition to being fluorogenic, this derivatization method also has the crucial advantage of being chromogenic (colorless <=> fuchsia). Chemical quantum calculations give an insight into the dye's molecular properties, while the development of an LC analytical method provides proof of concept regarding its application for the analysis of primary amines in a complex matrix.

1. Introduction

Modern analytical chemistry continuously pushes the limits of ultrasensitive analysis towards lower levels of detection [1]. Among all sensing methods, fluorescence spectroscopy appears to be the technique of choice because of its non-destructive character and its high sensitivity of response, especially for an extremely low concentration of analyte ($<10^{-9}$ M)[2,3]. Traditionally, there are two approaches for fluorescence-based sensing. One consists in turning on the fluorescence in the presence of a specific analyte while the other is to turn it off. Unequivocally, the turn-on method is significantly superior due to its high contrast to the background and the negligible risk of false positives, since most organic compounds are not intrinsically fluorescent [4].

In the case of a turn-on sensing, the simplest strategy is the fluorescence derivatization, *i.e.* the transformation of a non-emissive analyte into a fluorescent molecule, based in most cases on a chemical reaction [5]. The most frequently used methodology is the fluorescent labeling corresponding to the covalent attachment of an emissive dye (Scheme 1). The strong extrinsic fluorescence – brought by the coupled bright fluorophore – allows an efficient detection of the analyte with high sensitivity [6].



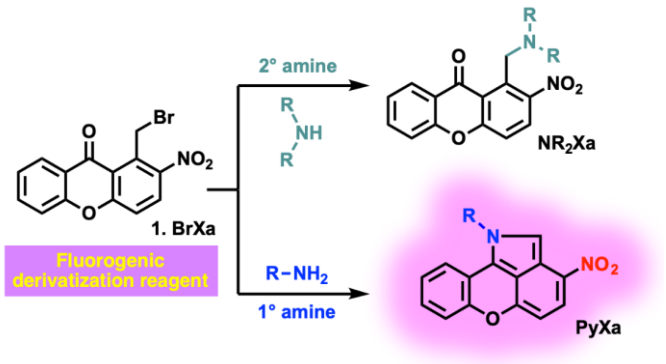
Scheme 1. State-of-the-art labeling for the amine fluorescent derivatization *vs.* rational design for the engineering of the targeted fluorogenic reagent **1**. *LG denotes leaving group.*

A myriad of fluorescent derivatization reagents has been synthesized; many of them being employed for quantitative purposes in analytical chemistry, generally for chromatography-based separations where exquisite sensitivity is required [7–10]. The derivatization and sensing of amines have been the subject of intense research as this functional group is found in many

biomolecules including amino acids, proteins and nucleic acids [11–17]. A wide variety of reagents for amine derivatization was reported, mainly based on the classical families of dyes (coumarin [18–20], Bodipy [21–23], fluorescein [24,25], rhodamine [26], cyanine [27]) bearing an amine-reactive function such as succinimyl and sulfosuccinimidyl esters (Scheme 1). To a lower extent, carbonyl azides, isothiocyanates, sulfonylchlorides and tetrafluorophenyl esters were proposed as an alternative amine-reactive group [7].

A major drawback of this technique is the fluorescence cross-contamination due to the large excess of the derivatization reagent used to drive the reaction kinetics to completion. Classically, this excess must either be chromatographically separated or removed by some pre-treatment procedures, as the intrinsic fluorescence of the reagent will interfere with both absorption and emission of the derivatized analyte and may contaminate the resulting fluorescent signal.

To overcome this, the ideal solution would be a "fluorogenic" reagent, *viz.* which fluoresces only after the derivatization reaction, thus avoiding both cross-contamination during the spectroscopic measurements and the implementation of extensive purification processes [28–33]. In that respect, innovative reagents such as quinolizinium [34], thiazolo-isoquinoline [35], DOOB – 2,2-diphenyl-1-oxa-3-oxonia-2-boratanaphthalene [36], catechol [37], stilbene trifluoroacetate [38], and ylidenemalononitrile enamines [39] were alternatively developed to sense fluorogenically various types of amines by derivatization. For that purpose, the most widely used fluorogenic derivatization reagent is *ortho*-pthalaldehyde, which intrinsically exhibits no fluorescence [40]. In the presence of 2-mercaptoethanol, it reacts chemoselectively with primary amines to afford emissive isoindoles [40–42]. The requirement for a large excess of this thiol additive combined with the blue fluorescence of the product, severely limits the usage of this system; especially for derivatization of amino-based biomolecules accompanied by strong background fluorescence. Indeed, besides demonstrating negligible fluorescence in the emission range of the derivatization reaction product, a fundamental property of a successful reagent should be its ability to emit an intense signal in the red or NIR region to avoid the auto-fluorescence from biological media. This could be achieved either through the significant rearrangement of the reagent scaffold or via its extension with a conjugate π-system [43]. It is expressly on this precise point of "fluorogenicity" that our research program has focused [44–46].



Scheme 2. Fluorogenic sensing using chemoselective reactivity of the derivatization reagent **1** towards primary amines. *Donor and acceptor groups involved in the push–pull relationship are depicted in blue and red, respectively.*

Previously, we reported the synthesis and biological activities of a series of pyrroloxanthones exhibiting substantial cytotoxicity against several cancer cell lines [47,48]. Interestingly, these compounds of the general formula **PyXa**, showed a bright fuchsia color in contrast to their starting material with a *pseudo* benzyl bromide **1** (**BrXa**), which presents the dramatic advantage of being completely colorless (Scheme 2). Noticeably, the Bischler-type reaction employed for the synthesis of **PyXa** proceeds only with primary amines, since secondary amines afford the amino-substituted compounds $\mathbf{NR_2X_2}$, with no color and fluorescence detected.

Prompted by these findings, we decided to further explore the potential of the above-mentioned scaffold as a fluorescent derivatization reagent for the colorimetric/fluorometric detection of primary amines. The chromogenic and fluorogenic sensing method would rely on the *in-situ* formation of an expanded pyrrole ring, thus extending the conjugation and concomitantly introducing a push–pull relationship between the electron-donating nitrogen of the sensed amine and the electron-withdrawing nitro group (Scheme 2). Therefore, the imposed structural changes are expected to shift the absorption maximum towards the red region of the electromagnetic spectrum, rendering **BrXa** an ideal candidate for amine derivatization. Additionally, the architecture obtained with a Donor-π-Acceptor (D-π-A) relationship should allow a larger dipole moment to be established in the excited state, which should be reflected in an enhanced sensitivity with the increasing polarity, resulting in a λ-shift to the lower energetic wavelength. This operating redshift should greatly facilitate amine detection by avoiding potential fluorescent cross-contamination from the extracts.

Herein, we report the synthesis of a novel xanthene-based reagent **BrXa** for the fluorogenic derivatization of primary amines. The implementation of this derivatization reaction will be described, as well as the photophysics of the product *i.e.* the newly formed fluorescent pyrroloxanthone. Its application to the derivatization of cyclopropylamine by the development of an LC analytical method will be provided. Correlation between the establishment of a push–pull relationship into the derivatized amine scaffold and the resulting environment sensitivity will be further evidenced by employing quantum mechanics calculations.

2. Experimental section

2.1. Material and methods

All commercially available chemicals and solvents were purchased from Alfa Aesar and used as received without any further purification. Melting points were determined on a Büchi apparatus and were uncorrected. All NMR spectra (^{1}H, ^{13}C, 2D) were recorded on 200, 400 or 600 MHz Bruker spectrometers respectively AC, Avance™ DRX and III instruments (Bruker BioSpin GmbH – Rheinstetten, Germany). ^{1}H-NMR (200, 400 and 600 MHz) and ^{13}C{^{1}H}NMR (50, 101 and 151 MHz, recorded with complete proton decoupling) spectra were obtained with samples dissolved in $CDCl_3$ or DMSO-d_6 with the residual solvent signals used as internal references: 7.26 ppm for C*H*Cl_3, and 2.50 ppm for $(CD_3)(CD_2H)S(O)$ regarding ^{1}H-NMR experiments; 77.2 ppm for $CDCl_3$ and 39.4 ppm for $(CD_3)_2S(O)$ concerning ^{13}C-NMR experiments [49,50]. Chemical shifts (δ) are given in ppm to the nearest 0.01 (^{1}H) or 0.1 ppm (^{13}C). The coupling constants (J) are given in Hertz (Hz). The signals are reported as follows: (s = singlet, d = doublet, t = triplet, m = multiplet, br = broad). Assignments of ^{1}H and ^{13}C-NMR signals were unambiguously achieved with the help of D/H exchange and 2D techniques: COSY, NOESY, HMQC, and HMBC experiments. Systematic xanthene nomenclature is used below for the assignment of each

spectrum. Flash chromatography was performed on Merck silica gel (40–63 μm) with the indicated solvent system using gradients of increasing polarity in most cases (Merck KGaA – Darmstadt, Germany) [51]. The reactions were monitored by analytical thin-layer chromatography (Merck pre-coated silica gel 60 F254 TLC plates, 0.25-mm layer thickness). Compounds were visualized on TLC plates by both UV radiation (254 and 365 nm) and spraying with a staining agent (vanillin, PMA, $KMnO_4$ or ninhydrin) followed by subsequent warming with a heat gun. All solvents for absorption and fluorescence experiments were of spectroscopic grade. Absorption spectra were recorded on a Cary 100 Bio UV–Vis spectrophotometer (Varian/Agilent) using Suprasil® quartz cuvettes with 1-cm path length. Stock solutions of investigated dyes were prepared using dimethylformamide. The samples used for spectroscopic measurements contained ≈ 0.2% *v/v* of solvents of the stock solution. Fluorescence spectra were recorded on a FluoroMax 4.0 spectrofluorometer (Jobin Yvon, Horiba) with a thermostatically controlled cell compartment at 20 ± 0.5 °C with slits open to 2 nm and were corrected for Raman scattering, lamp fluctuations and instrumental wavelength-dependent bias. Excitation wavelengths were set at the absorption maxima except when mentioned in the corresponding experiments. A Waters Acquity UPLC system (Waters Corporation – Manchester, UK), comprising a solvent manager module connected to an autosampler, was employed for the analytical chromatography. Spectrophotometric detection was performed using an Acquity PDA detector. Chromatographic separation was achieved on a C18 BEH column (Waters Acquity, 50 mm × 2.1 mm, 1.7 m). Mass spectra were recorded on a hybrid LTQ™ Orbitrap Discovery XL instrument (Thermo Fisher Scientific – Bremen, Germany), coupled to an Accela HPLC system (Thermo Fisher Scientific) equipped with a binary pump, an autosampler, and Xcalibur 2.1 as a software.

*2.2. Ethyl 2-iodobenzoate (**3**)*

A suspension of 2-iodobenzoic acid **2** (50 g, 0.20 mol) and conc. H_2SO_4 (4.5 mL) in ethanol (200 mL) was refluxed for 24 h. The progress of the reaction was monitored using TLC. After completion of the reaction, volatiles were removed under reduced pressure and the resulting mixture was diluted with dichloromethane (500 mL) and neutralized with 10% Na_2CO_3 (50 mL). The organic layer was separated using a separating funnel, then dried over Na_2SO_4 and evaporated to dryness to afford 52 g (94 %) of the ester **3**, which was used without further purification for the next reaction. MS (ESI^+, MeOH): *m/z*: 277.0 $[M+H]^+$.

*2.3. Ethyl 2-(3-methylphenoxy)benzoate (**5**)*

A suspension of *m*-cresol **4** (35.55 g, 0.33 mol), **3** (45.43 g, 0.17 mol), K_2CO_3 (45.33 g, 0.33 mol) and CuCl (6.45 g, 0.028 mol) in dry pyridine (250 mL) was heated at 120 °C for 24 h under argon atmosphere. After completion of the reaction as indicated by TLC, volatiles were removed under reduced pressure and the residue was diluted in CH_2Cl_2 (100 mL). The organic layer was washed subsequently with 3M aq. HCl solution (3 x 50 mL), water (3 x 50 mL), brine (3 x 50 mL), dried over Na_2SO_4, and evaporated to dryness. Flash chromatography on silica gel using a mixture of cyclohexane/EtOAc (20:1, v/v) afforded **5** as an oil (40.04 g, 92 %). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.21 (t, *J* = 7.1 Hz, 3H, C*H*$_3$CH$_2$), 2.29 (s, 3H, CH_3), 4.25 (q, *J* = 7.1 Hz, 2H, C*H*$_2$CH$_3$), 6.72–6.75 (m, 2H, H-2′, H-6′), 6.86 (d, *J* = 7.6 Hz, 1H, H-4′), 6.98 (dd, *J* = 8.3, 0.8 Hz, 1H, H-3), 7.16 (m, 2H, H-5′, H-5), 7.43 (td, *J* = 8.3, 1.8 Hz, H-4), 7.9 (dd, *J* = 7.8, 1.8 Hz, 1H, H-6). ^{13}C-NMR (50 MHz, $CDCl_3$): δ 14.1 (*C*H$_3$CH$_2$), 21.4 (CH_3), 61.0 (CH$_3$*C*H$_2$), 114.9 (C-6'), 118.5 (C-2'), 121.2 (C-3), 123.6 (C-5), 123.7 (C-4'), 123.9 (C-1), 129.4 (C-5'), 131.8 (C-6), 133.5 (C-4), 139.7 (C-3'), 155.9 (C-1'), 157.9 (C-2), 165.6 (CO). MS (ESI^+, MeOH): *m/z*: 257.1 $[M+H]^+$; HRMS (ESI^+): *m/z* calcd for $C_{16}H_{17}O_3$: 257.1172 $[M+H]^+$; found 257.1177.

*2.4. Ethyl 2-(3-methyl-4-nitrophenoxy)benzoate (**6b**)*

To a suspension of ester **5** (39.46 g, 0.15 mol) in acetic anhydride (93.4 ml, 0.99 mol), previously cooled down to 0 °C, was added dropwise a solution of fuming HNO_3 (6.7 ml, 0.16 mol) in acetic anhydride (23.3 ml, 0.25 mol). The resulting mixture was stirred at rt for 24 h. After completion of the reaction, the mixture was poured into ice-water bath, basified with 15% NaOH aq. solution (pH ~ 9) and extracted with CH_2Cl_2 (3 x 30 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was purified by column chromatography on silica gel, using petroleum ether/EtOAc (30:1), to afford **6a** as the major regioisomer (29.28 g, 63 %) and **6b** (9.76 g, 21 %) as the minor one. *Ethyl 2-(5-methyl-2-nitrophenoxy)benzoate* **6a**: oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.16 (t, *J* = 7.1 Hz, 3H, CH$_2$C*H*$_3$), 2.30 (s, 3H, CH_3), 4.22 (q, *J* = 7.1 Hz, 2H, C*H*$_2$CH$_3$), 6.58 (s, 1H, H-6′), 6.95 (d, *J* = 8.3 Hz, 1H, H-4′), 7.11 (d, *J* = 8.2 Hz, 1H, H-3), 7.33 (td, *J* = 8.2, 2.1 Hz, 1H, H-5), 7.58 (td, *J* = 8.2, 2.1 Hz, 1H, H-4), 7.92 (d, *J* = 8.3 Hz, 1H, H-3′), 8.03 (dd, *J* = 8,2, 2.1 Hz, 1H, H-6). ^{13}C-NMR (50 MHz, $CDCl_3$): δ 13.8 (CH$_2$*C*H$_3$), 21.6 (CH_3), 61.2 (*C*H$_2$CH$_3$), 118.5 (C-6′), 122.1 (C-3), 123.1 (C-4′), 124.0 (C-1), 125.2 (C-5), 125.8 (C-3′), 132.3 (C-6), 133.9 (C-4), 137.8 (C-2′), 145.9 (C-5′), 151.7 (C-1′), 153.8 (C-2), 164.9 (CO). **6b**: white solid, m.p. = 63–64 °C (Et_2O/*n*-hexane). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.18 (t, *J* = 7.3 Hz, 3H, CH$_2$C*H*$_3$), 2.56 (s, 3H, CH_3), 4.20 (q, *J* = 7.3 Hz, 2H, C*H*$_2$CH$_3$), 6.75 (d, *J* = 8.2 Hz, 1H, H-6′), 6.8 (s, 1H, H-2′), 7.15 (d, *J* = 8.3 Hz, 1H, H-3), 7.36 (t, *J* = 8.3 Hz, 1H, H-5), 7.62 (t, *J* = 8.3 Hz, 1H, H-4), 8.03 (d, *J* = 8.3 Hz, 1H, H-6), 8.05 (d, *J* = 8.2 Hz, 1H, H-5′). ^{13}C-NMR (50 MHz, $CDCl_3$): δ 14.0 (CH$_2$*C*H$_3$), 21.5 (CH_3), 61.2 (*C*H$_2$CH$_3$), 113.9 (C-6′), 119.4 (C-2′), 123.1 (C-3), 124.5 (C-1), 125.7 (C-5), 127.4 (C-5′), 132.3 (C-6), 134.1 (C-4), 137.0 (C-3′), 143.1 (C-4′), 153.4 (C-2), 162.0 (C-1′), 164.7 (CO). MS (ESI^+, MeOH): *m/z*: 302.1 $[M+H]^+$; HRMS (ESI^+): *m/z* calcd for $C_{16}H_{16}NO_5$: 302.1023 $[M+H]^+$; found 302.1019.

*2.5. 2-(3-Methyl-4-nitrophenoxy)benzoic acid (**7**)*

To a solution of ester **6b** (9.4 g, 30 mmol) in ethanol at rt, a cold 15% NaOH aq. solution (10 mL, 100 mmol) was added dropwise. The reaction mixture was stirred at rt for 1.5 h, before being poured into water and acidified with 6M HCl aq. solution (pH ~ 2). The resulting white precipitate was filtered and dried over phosphorus pentoxide (glass dessicator) to afford **7** (8.13 g, 96 %), which was used for the next step without any further purification. M.p. = 144–145 °C (EtOAc). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.43 (s, 3H, CH_3), 6.90 (s, 1H, H-2′), 6.94 (d, *J* = 8.2 Hz, 1H, H-6′), 7.15 (d, *J* = 8.1 Hz, 1H, H-3), 7.26 (t, *J* = 8.1 Hz, 1H, H-5), 7.57 (td, *J* = 8.1, 1.9 Hz, H-4), 8.04 (d, *J* = 8.2 Hz, 1H, H-5′), 8.22 (dd, *J* = 8.1, 1.9 Hz, H-6). ^{13}C-NMR (50 MHz, $CDCl_3$): δ 21.7 (CH_3), 120.2 (C-6′), 120.7 (C-2'), 121.4 (C-3), 124.7 (C-1), 124.8 (C-5), 126.1 (C-5′), 133.3 (C-6), 135.0 (C-4), 138.6 (C-3′), 146.6 (C-4′), 150.1 (C-2), 155.6 (C-1'), 168.9 (CO). MS (ESI^+, MeOH): *m/z*: 274.1 $[M+H]^+$; HRMS (ESI^+): *m/z* calcd for $C_{14}H_{12}NO_5$: 274.0710 $[M+H]^+$; found 274.0713.

*2.6. 1-Methyl-2-nitro-9H-xanthen-9-one (**8**)*

A suspension of acid **7** (6.20 g, 0.023 mol) in polyphosphoric acid (15 mL) was stirred at 100 °C for 2 h. After cooling, the mixture was poured into crushed-ice bath. The resulting precipitate was filtered, washed with 10% Na_2CO_3 solution and water, and air-dried. Flash chromatography on silica gel, using a mixture of cyclohexane/CH_2Cl_2 (12:1 → 5:1, *v/v*) afforded **8** as a white solid

(5.4 g, 92 %). M.p. = 206–207 °C (EtOH) (lit. 205–207 °C) [47]. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 2.85 (s, 3H, CH_3), 7.50 (td, J = 7.9, 0.8 Hz, 1H, H-7), 7.64 (d, J = 9.0 Hz, 1H, H-4), 7.68 (d, J = 7.9 Hz, 1H, H-5), 7.88 (td, J = 7.9 Hz, 0.8 Hz, 1H, H-6), 8.13 (dd, J = 7.9, 0.8 Hz, 1H, H-8), 8.23 (d, J = 9.0 Hz, 1H, H-3). ^{13}C-NMR (50 MHz, DMSO-d_6): δ 16.9 (CH_3), 118.0 (C-5), 118.1 (C-4), 120.1 (C-9a), 122.2 (C-8a), 125.2 (C-7), 126.4 (C-8), 129.6 (C-3), 135.4 (C-1), 136.0 (C-6), 147.6 (C-2), 154.5 (C-10a), 158.5 (C-4a), 177.7 (CO). MS (ESI$^+$, MeOH): *m/z*: 256.1 [M+H]$^+$; HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{10}NO_4$: 256.0604 [M+H]$^+$; found 256.0609.

*2.7. 1-(bromomethyl)-2-nitro-9H-xanthen-9-one (**1** – **BrXa**)*

A suspension of xanthone **8** (2.86 g, 11 mmol), NBS (1.96 g, 11 mmol) and BPO (dibenzoyl peroxide, 286 mg, 1.18 mmol) in CCl_4 (100 mL) was refluxed for 8 h under UV irradiation (compact fluorescent lamp – CFL 150 W). After completion of the reaction, the mixture was washed with 5% $NaHCO_3$ aq. solution (3 x 20 mL), 5% $Na_2S_2O_4$ aq. solution (3 x 20 mL), water (3 x 50 mL), dried over Na_2SO_4, and evaporated to dryness. Flash chromatography on silica gel using a mixture of cyclohexane/CH_2Cl_2 (10:1 → 5:1, *v/v*) as the eluent afforded **1** (3.01 g, 82 %). M.p. = 179–181 °C (EtOH) [47]. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.29 (s, 2H, CH_2), 7.41–7.48 (m, 2H, H-7, H-5), 7.53 (d, J = 8.4 Hz, 1H, H-4), 7.77 (td, J = 8.7, 1.7 Hz, 1H, H-6), 8.17 (d, J = 8.4 Hz, 1H, H-3), 8.29 (dd, J = 8.7, 1.7 Hz, 1H, H-8). ^{13}C-NMR (50 MHz, $CDCl_3$): δ 23.3 (CH_2Br), 117.6 (C-5), 119.0 (C-9a), 120.3 (C-4), 122.8 (C-8a), 125.1 (C-7), 127.2 (C-8), 129.8 (C-3), 135.3 (C-6), 136.2 (C-1), 146.5 (C-2), 155.5 (C-10a), 159.6 (C-4a), 177.8 (CO). MS (ESI$^+$, MeOH): *m/z*: 334.0, 336.0 [M+H]$^+$; HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_9BrNO_4$: 333.9709 [M+H]$^+$; found 333.9711.

*2.8. 1-butyl-3-nitro-1H-chromeno[4,3,2-cd]isoindole (**9**)*

To a suspension of compound **1** (35 mg, 0.10 mmol) in dry MeOH (2 mL), *n*-butylamine (20 eq.) was added. The resulting mixture was stirred at rt for 2 h and the progress of the reaction was monitored by TLC. After the completion of the reaction, the precipitate was filtered off and then recrystallized with CH_2Cl_2/Et_2O to afford **9** (29 mg, 94 %). M.p. 198–199 °C (CH_2Cl_2/Et_2O). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.94 (t, J = 7.3 Hz, 3H, CH_3), 1.35–1.49 (m, 2H, $CH_2CH_2CH_3$), 1.85–1.97 (m, 2H, $CH_2CH_2CH_3$), 4.40 (d, J = 7.4 Hz, 2H, N-CH_2), 6.11 (d, J = 8.4 Hz, 1H, H-5), 7.14–7.25 (m, 3H, H-7, H-9, H-8), 7.40 (s, 1H, H-2), 7.48 (dd, J = 7.6, 1.5 Hz, 1H, H-10), 8.07 (d, J = 8.4 Hz, 1H, H-4). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 13.7 (CH_3), 19.9 ($CH_2CH_2CH_3$), 32.8 ($CH_2CH_2CH_3$), 50.0 (N-CH_2), 98.1 (C-5), 115.0 (C-2a), 116.4 (C-2), 118.2 (C-10a), 118.6 (C-7), 119.3 (C-10b), 120.5 (C-2a^1), 120.5 (C-10), 125.3 (C-9), 128.0 (C-8), 130.1 (C-4), 131.7 (C-3), 152.9 (C-6a), 157.6 (C-5a). MS (ESI$^+$, MeOH): *m/z*: 309.1 [M+H]$^+$; HRMS (ESI$^+$): *m/z* calcd for $C_{18}H_{17}N_2O_3$: 309.1234 [M+H]$^+$; found 309.1233.

*2.9. 1-dodecyl-3-nitro-1H-chromeno[4,3,2-cd]isoindole (**10**)*

This compound **10** was synthesized following a similar procedure as described for **9**, using dodecacylamine as the proper amine of the reaction. Yield: 95 %. M.p. 113–114 °C (CH_2Cl_2/Et_2O). ^{1}H-NMR (600 MHz, $CDCl_3$): δ 0.87 (t, J = 7.3 Hz, 3H, CH_3), 1.25–1.30 (m, 12H, dodecyl), 1.35 (m, 2H, dodecyl), 1.44 (p, J = 7.3 Hz, 2H, dodecyl), 1.56–1.66 (m, 2H, dodecyl), 1.94–2.01 (m, 2H, dodecyl), 4.44 (t, J = 7.4 Hz, 2H, N-CH_2), 6.19 (dd, J = 8.6, 1.8 Hz, 1H, H-5), 7.19–7.26 (m, 2H, H-7, H-9), 7.29 (dd, J = 8.1, 1.7 Hz, 1H, H-8), 7.47 (s, 1H, H-2), 7.53 (dd, J = 7.7, 1.8 Hz, 1H, H-10), 8.15 (dd, J = 8.6, 1.7 Hz, 1H, H-4). ^{13}C-NMR (151 MHz, $CDCl_3$): δ 14.23 (CH_3), 22.8 (dodecyl), 26.8 (dodecyl), 29.3 (dodecyl), 29.5 (dodecyl), 29.5 (dodecyl), 29.7 (2 x dodecyl), 29.7 (dodecyl), 31.0 (dodecyl), 32.0 (dodecyl), 50.5 (N-CH_2), 98.2 (C-5), 115.3 (C-2a), 116.5 (C-2), 118.5 (C-10a), 118.8 (C-7), 119.4 (C-10b), 120.6 (C-2a^1), 120.6 (C-10), 125.4 (C-9), 128.1 (C-8), 130.2 (C-4), 132.1 (C-3), 153.2 (C-6a), 157.7 (C-5a). MS (ESI$^+$, MeOH): *m/z*: 421.2 [M+H]$^+$; HRMS (ESI$^+$): *m/z* calcd for $C_{26}H_{33}N_2O_3$: 421.2486 [M+H]$^+$; found 421.2490.

*2.10. 1-cyclopropyl-3-nitro-1H-chromeno[4,3,2-cd]isoindole (**11**)*

This compound **11** was synthesized following a similar procedure as described for **9**, using cyclopropylamine as the proper amine of the reaction. Yield: 93 %. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.31–1.41 (m, 4H, CH_2), 3.80 (m, 1H, CH-cyclopropyl), 6.18 (d, J = 8.3 Hz, 1H, H-5), 7.20–7.33 (m, 3H, H-7, H-9, H-8), 7.54 (s, 1H, H-2), 8.04 (dd, J = 7.4, 1.8 Hz, 1H, H-10), 8.15 (d, J = 8.3 Hz, 1H, H-4). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 8.9 (CH_2), 31.3 (CH-cyclopropyl), 98.1 (C-5), 114.7 (C-2a), 115.7 (C-2), 118.3 (C-10a), 118.3 (C-7), 120.1 (C-10b), 121.5 (C-2a^1), 121.8 (C-10), 125.0 (C-9), 128.0 (C-8), 130.5 (C-4), 131.9 (C-3), 153.0 (C-6a), 157.7 (C-5a). MS (ESI$^+$, MeOH): *m/z*: 293.1 [M+H]$^+$; HRMS (ESI$^+$): *m/z* calcd for $C_{17}H_{13}N_2O_3$: 293.0921 [M+H]$^+$; found 293.0923.

*2.11. 1-((diethylamino)methyl)-2-nitro-9H-xanthen-9-one (**12**)*

This compound **12** was synthesized following a similar procedure as described for **9**, using diethylamine as the proper amine of the reaction. Yield: 89 %. ^{1}H-NMR (600 MHz, $CDCl_3$): δ 0.90 (t, J = 7.1 Hz, 6H, CH_2CH_3), 2.47 (q, J = 7.1 Hz, 4H, CH_2CH_3), 4.70 (s, 2H, CH_2), 7.41 (dd, J = 8.1, 1.1 Hz, 1H, H-5), 7.44–7.48 (m, 2H, H-7, H-4), 7.74 (dd, J = 8.1, 1.1 Hz, 1H, H-6), 7.82 (d, J = 9.0 Hz, 1H, H-3), 8.26 (dd, J = 8.0, 1.1 Hz, 1H, H-8). ^{13}C-NMR (151 MHz, $CDCl_3$): δ 11.2 (CH_2CH_3), 46.8 (CH_2CH_3), 50.3 (CH_2), 117.6 (C-5), 117.9 (C-9a), 121.0 (C-4), 122.8 (C-8a), 124.8 (C-7), 127.2 (C-8), 129.2 (C-3), 135.4 (C-6), 141.3 (C-1), 148.5 (C-2), 154.9 (C-10a), 157.8 (C-4a), 178.5 (CO). MS (ESI$^+$, MeOH): *m/z*: 327.1 [M+H]$^+$; HRMS (ESI$^+$): *m/z* calcd for $C_{18}H_{19}N_2O_4$: 327.1339 [M+H]$^+$; found 327.1334.

*2.12. 3-nitro-1-(2-(pyrrolidin-1-yl)ethyl)-1H-chromeno[4,3,2-cd]isoindole (**13**)*

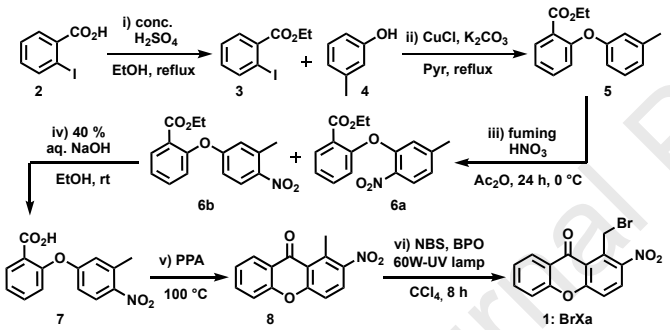
To a suspension of **1** (70 mg, 0.20 mmol) in abs. EtOH (3 mL), was added 2-propylaminoethylamine (20 eq.). The reaction mixture was stirred at rt for 2 h, before being reduced under vacuum, taken over with CH_2Cl_2, washed with H_2O, dried over Na_2SO_4, and filtered. The volatiles were removed *in vacuo* and the resulting crude was purified by flash chromatography on silica gel eluted with CH_2Cl_2/MeOH (97:3, v/v) to afford **13** as an oil (66 mg, 94 %) [48]. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.76 (br. s, 4H, (CH_2CH_2)$_2$N-), 2.56 (br. s, 4H, (CH_2CH_2)$_2$N), 2.91 (t, J = 7.5 Hz, 2H, (CH_2CH_2)$_2$NCH_2), 4.45 (t, J = 7.5 Hz, 2H, (CH_2CH_2)$_2$NCH$_2$CH_2), 6.00 (d, J = 8.3 Hz, 1H, H-5), 7.15–7.17 (m, 3H, H-7, H-9, H-8), 7.32 (s, 1H, H-2), 7.48 (d, J = 7.3 Hz, 1H, H-10), 7.97 (d, J = 8.3 Hz, 1H, H-4). ^{13}C-NMR (50 MHz, $CDCl_3$): δ 23.6 ((CH_2CH_2)$_2$NCH$_2$CH$_2$), 49.4 ((CH_2CH_2)$_2$NCH$_2$$CH_2$), 54.5 (($CH_2CH_2$)$_2NCH_2CH_2$), 56.3 (($CH_2CH_2$)$_2NCH_2CH_2$), 98.0 (C-5), 115.0 (C-2a), 116.4 (C-2), 117.9 (C-10a), 118.5 (C-7), 119.5 (C-10b), 120.1 (C-2a^1), 120.5 (C-10), 125.3 (C-9), 128.0 (C-8), 130.1 (C-4), 131.6 (C-3), 152.8 (C-6a), 157.5 (C-5a). MS (ESI$^+$, MeOH): *m/z*: 350.1 [M+H]$^+$; HRMS (ESI$^+$): *m/z* calcd for $C_{20}H_{20}N_3O_3$: 350.1499 [M+H]$^+$; found 350.1496.

3. Results and discussion

3.1. Preparation of the derivatization reagent

Our efforts were initially focused on the development of a reliable procedure for the scalable synthesis of the proposed reagent **BrXa**. To overcome previous difficulties such as the number of steps and overall yield [47,48], we considered a more convergent approach based on a copper-catalyzed coupling reaction (Scheme 3).

Commercially available 2-iodobenzoic acid **2** was used as a starting material which, upon esterification in ethanol, yielded almost quantitatively the corresponding ethyl ester **3**. Ullmann condensation of *m*-cresol **4** with the aryl iodide **3** efficiently afforded the coupling product **5**. Nitric acid treatment in acetic anhydride of the biaryl ether **5**, led to a mixture of nitro-isomeric compounds **6a** and **6b**, separable by silica gel chromatography and identifiable by 2D-NMR. More specifically, structural discrimination resulted from the observation of ^{3}J couplings between the methyl group and two aromatic CH carbons in *ortho* in the case of **6a**, against a single correlation for **6b**. Subsequently, saponification under mild conditions of the ester **6b** provided the carboxylic acid **7**, which was ring-closed upon treatment with PPA to give the xanthone **8**. The bromomethyl target **1** was finally obtained from **8** upon free-radical bromination with NBS under UV irradiation.

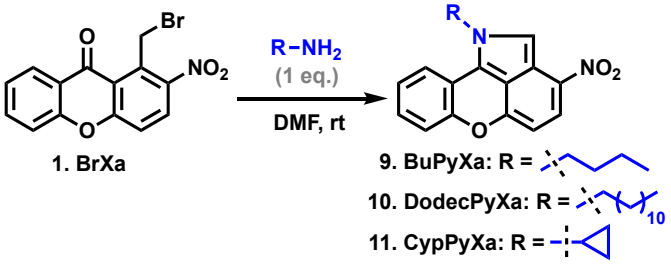


Scheme 3. Synthetic pathway leading to the derivatization reagent **BrXa**.

It is worth mentioning that the overall yield of the newly developed synthesis is >40 %, *i.e.*, thrice higher than the precedent approach. In a nutshell, an original and straightforward synthetic approach has been developed for the preparation of the derivatization reagent **BrXa**, using practical, inexpensive and scalable procedures with purification processes remaining simple.

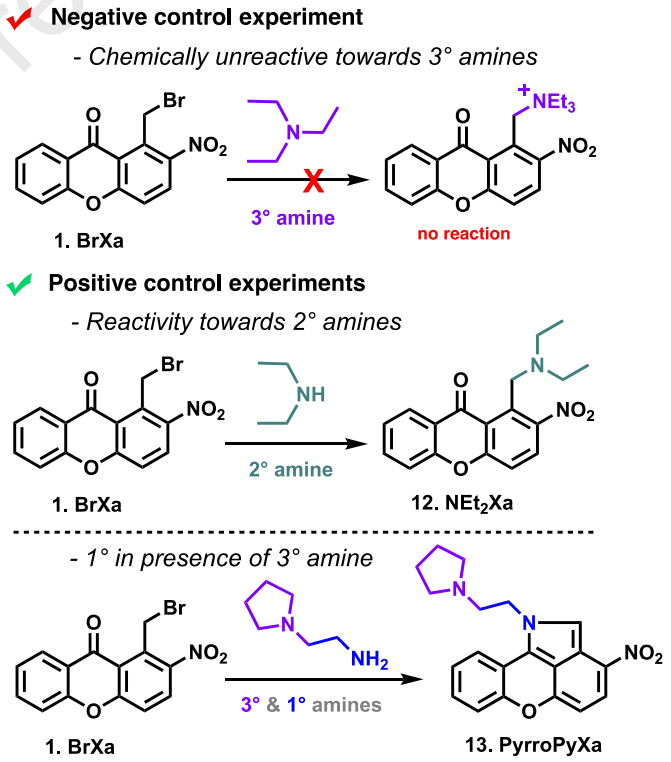
3.2. Derivatization reaction

To develop suitable conditions for this derivatization in order to make it an effective detection tool, a certain set of specifications have to be met. First of all, the reaction must be performed at room temperature to be truly practical. Another prerequisite is to find a solvent in which both the derivatization reagent **BrXa** and the targeted amines are fully soluble and also remains reactive. Protic solvents such as methanol and ethanol – typical for this kind of reaction – revealed to be not enough solvating in the concentration range routinely used for spectroscopic measurements. The chosen solvent has to be polar to allow the first step (S_N2) to be conducted, but the *pseudo* benzylic bromide – known to be reactive – must be stable during the reaction time. Dimethylformamide proved to be the one that ticks all the boxes.



Scheme 4. Implementation of the derivatization reaction for primary amines including aliphatic and saturated cyclic R-NH_2 derivatives.

Using DMF as a solvent at room temperature, first attempts on selected primary amines were carried out in batch. Derivatization reaction proceeded smoothly on aliphatic amines with side chains of different sizes (*butyl* and *dodecyl*) to quantitatively furnish the corresponding pyrroloxanthones, **9 BuPyXa** and **10 DodecPyXa**, respectively (Scheme 4). Several observations could be made: *i)* a clear magenta color appears along the derivatization process, offering an easy readout *ii)* a distinct vermilion fluorescence emerges upon excitation with any type of laser ranging from blue to yellow, allowing the sensitivity of the reaction to be substantially increased. As a consequence, these properties make the detection chromogenic and fluorogenic. Moreover, the simplicity of the method is clearly an asset, since there is no requirement of anhydrous conditions, temperature or precise order in the addition of the reagent.



Scheme 5. Negative *(top)* and positive *(bottom)* control experiments performed for the engineering of the derivatization reaction.

Then, to establish the selectivity towards the order (1°, 2° & 3°) of the amines to be derivatized, different types of amines were chosen. Triethylamine was first screened as a negative control, and as expected, no reaction was observed with this tertiary amine (Scheme 5). This chemical inertia bodes well for the development of analytical methods with amine-based buffers. Thereafter, the reactivity of secondary amines was tested using diethylamine. As suspected, the first S_N2-type step worked normally to provide **12**, but no further cyclization could take place due to the formation of

an unstable ammonium intermediate (Scheme S1). Advantageously, **NEt$_2$Xa** has an electronic π-scaffold similar to that of the reagent, meaning therefore that it will be both colorless and devoid of fluorescence. Thus, derivatized secondary amines should not cross-contaminate the colorimetric detection of the amines of interest during the derivatization of a complex matrix.

Eventually, using 2-(pyrrolidinyl)ethylamine explicitly showed both the lack of reactivity of tertiary amines and the versatility of primary R-NH$_2$ for this derivatization procedure. Thence, this test exclusively produced the pyrrolidinyl-pyrroloxanthone **13**, **PyrroPyXa**. Furthermore, a competition experiment using *N*-methylethylenediamine demonstrated the superior reactivity of the 1° amine at the expense of the 2° one towards **BrXa** (Fig. S7–9).

3.3. Chromatographic and mass spectrometry analysis

In order to apply the novel derivatization reagent to chromatographic analysis, cyclopropylamine was selected as a model analyte. The derivatization of the saturated cyclic primary amine with **BrXa** proceeded smoothly in acetonitrile to yield **11 CypPyXa**, as the derivatized product (Scheme 4). Triethylamine was used as a base to scavenge HBr formed *in situ* during the reaction process.

An analytical method was developed to separate the product from the excess of the derivatization reagent, using H$_2$O and acetonitrile, as solvents A & B, respectively. The total analysis time, including column equilibration, was 5 min per injection and the flow rate was set at 0.5 mL/min. The gradient elution program used was as follows: from 10% to 100% B in 3.0 min => 0.5 min at 100% B => from 100% to 10% B in 0.2 min => equilibration at 10% B for 1.3 min. The injection volume was adjusted to 5 μL and the column temperature was maintained at 40 °C throughout all experiments.

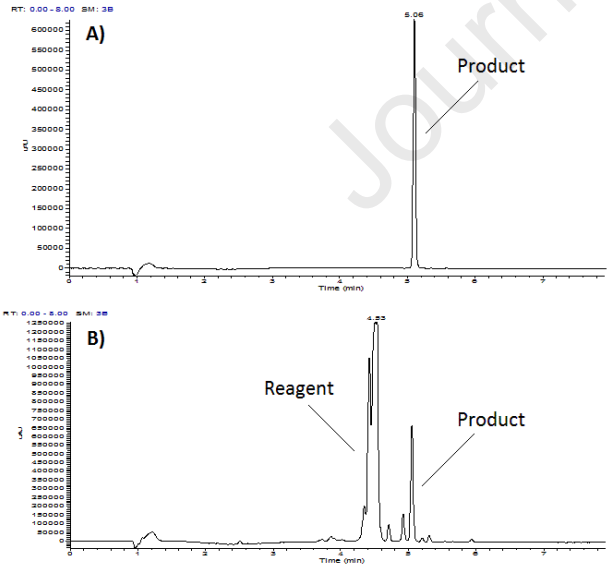


Fig. 1. Representative chromatograms of the HPLC purification process recorded by the PDA detector set at A) 537 nm and B) 280 nm.

The chromatograms depicted in Fig. 1 demonstrate that a baseline separation was obtained (Fig. 1B). The derivatized product is the only one that can be detected in the yellow range (Fig. 1A) due to its extended π-conjugation induced by the formation of an additional ring.

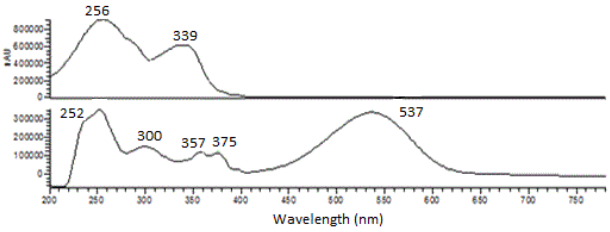


Fig. 2. UV–Vis spectra of the peaks, respectively assigned to the derivatization reagent **BrXa** (*top*) and the derivatized cyclopropylamine **CypNXa** (*bottom*). *Snapshots collected by the PDA detector.*

Absorbance spectra – illustrated in Fig. 2 – confirmed this increase of conjugation occurring through the derivatizing reaction, even in presence of a chromophore-free amine. Indeed, an intense and broad absorption band centered around 540 nm clearly appears, as a result of the important electronic conjugation being established between the pyrrolo donor (D) and nitro acceptor (A) groups. On another note, even in case of contamination by traces of reagent, these observables substantiate that no cross-excitation can take place given the λ-shift of *ca.* 200 nm offered by the derivatized product. This bodes well for the photophysical characterization of the sensing reaction.

Transferring this liquid chromatographic analysis to an MS detector provides a more reliable characterization. Both analytes were precisely identified in the presented MS spectra obtained from the respective chromatographic peaks (Fig. 3). Typically, the two molecular ion isotopes observed with similar abundance were attributed to the presence of a bromine in the reagent structure.

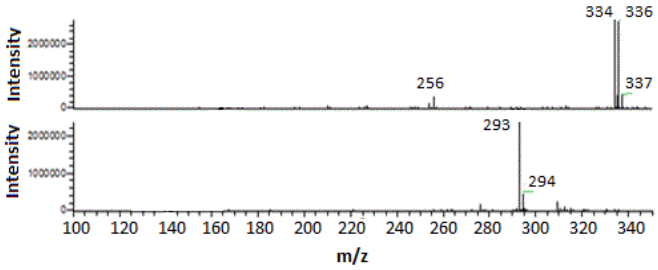


Fig. 3. Mass spectrograms of the peaks assigned to the derivatization reagent (*top*) and derivatized cyclopropylamine (*bottom*). *The expected theoretical m/z values for the [M+H]$^+$ molecular ions of both compounds are* respectively*:* **BrXa** *($C_{14}H_8O_4BrN$) = 333.971 & 335.969; and* **CypPyXa** *($C_{17}H_{12}O_2N_2$) = 293.092.*

3.4. Photophysical characterization

To study this derivatization reaction in detail from a photophysical point of view, cyclopropylamine (Cyp-NH$_2$) was selected again, this time for the electron-releasing character of its saturated three-membered ring. Indeed, this inductive +*I* effect should enhance the donor strength in the push–pull relationship operating in the excited state with the nitro acceptor.

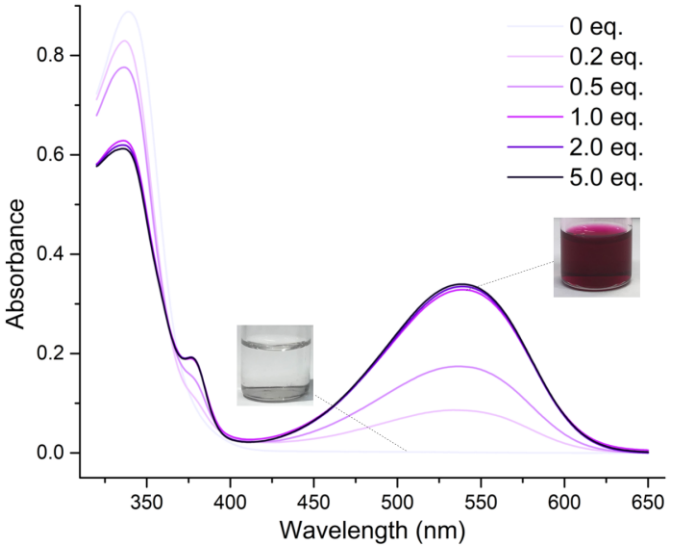
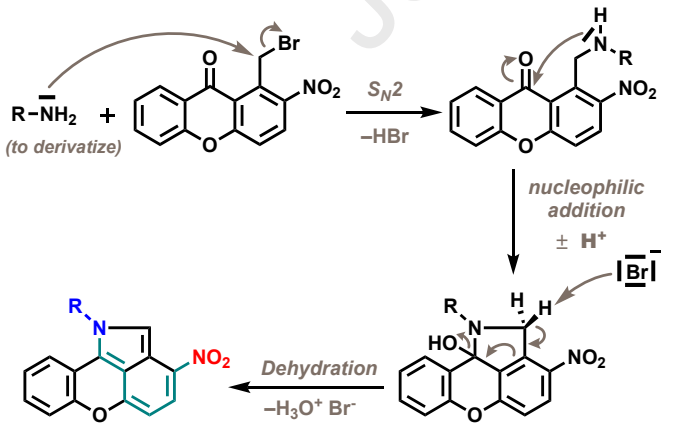


Fig. 4. Investigation of the colorimetric sensing: spectrophotometric titration of **BrXa** with Cyp-NH_2 leading to **CypPyXa**. Inserts represent the initial *(left)* and ending *(right)* solutions.

To check the chromogenic detection, the derivatization reagent was titrated with this challenging primary amine, and the formation of the resulting **CypPyXa** was monitored by UV–Vis spectroscopy (Fig. 4). For this purpose, **BrXa** was dissolved in DMF in the mM range, and changes in absorption were recorded upon the gradual addition of Cyp-NH_2. Since the xanthone core of **BrXa** absorbs at approx. 340 nm, and thus outside the visible range, the starting solution is completely colorless (*insert* in Fig. 4). As soon as the primary amine is added, an absorption band emerges in the yellow range (*ca.* 535 nm), transducing the appearance of the fuchsia color observed for the reaction mixture. Once an equivalent is reached, the yellow absorbance of **CypPyXa** no longer seems to change, and the solution turns into a persistent dark magenta (*insert* in Fig. 4). A similar observation could be made during the fluorescence titration where the emission signal does not fluctuate after addition of one equivalent of 1° amine (Figure S1). Therefore, these results highlight a 1: 1 stoichiometry for the derivatization reaction, as reflected by the mechanism hypothesized herein in Scheme 6.



Scheme 6. Hypothetical mechanism of the derivatization reaction using one equivalent of primary amine. *The push–pull relationship, involving the donor (blue) and acceptor (red) groups, is depicted in green.*

To provide further insights into the spectroscopic features of **CypPyXa**, absorption measurements were conducted in a set of solvents of increasing polarity, as transcribed by the Dimroth-Reichardt $E_T^N(30)$ parameter [52]. This empirical scale takes into account the dielectric constant and the H-bond donating ability of the solvent. It is normalized from TMS (0) to water (1). Absorption spectra displayed a certain solvatochromism (about 35 nm) since the maxima vary from 510 nm in toluene to 544 nm for DMSO (Table 1 & Fig. 5). This moderate dependency on solvent polarity revealed that charge transfer between the donor and acceptor groups is already operating in the ground state. This results in a color change from crimson red to purplish blue, respectively for apolar and strongly polar media. In protic EtOH, it is noteworthy that H-bonding with the NO_2 acceptor group induced a hypsochromic shift of the absorption band.

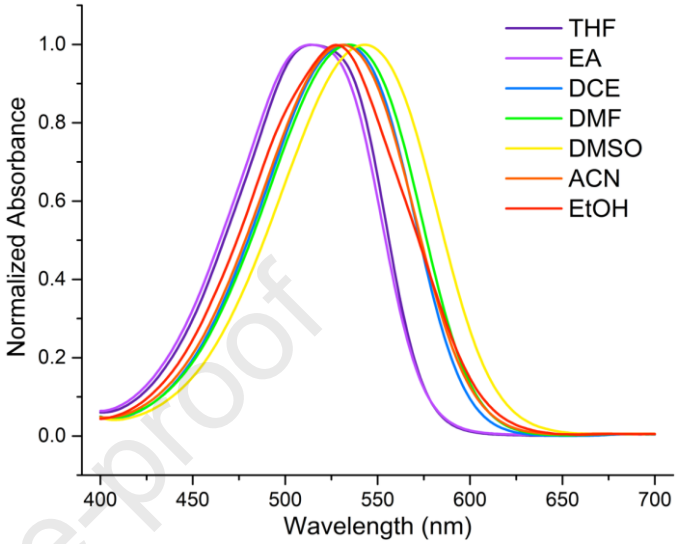


Fig. 5. Absorption observables of the derivatized adduct **CypPyXa**, illustrating solvatochromic characteristics.

Molar absorptivity of **CypPyXa** was determined to be approx. 13,000 $M^{-1}\cdot cm^{-1}$ in DMF, almost twice as high as that of **BrXa** (ε = 7,000 $M^{-1}\cdot cm^{-1}$). That observation is consistent with the extension of the π-system occurring during derivatization due to the pyrrole ring formation [43]. This augurs well for the fluorogenic sensing, given that brightness is the product of the molar extinction coefficient and the quantum yield.

Table 1. Photophysical properties of **CypPyXa** in various solvents, representative of a wide range of polarity.[a]

Solvent	$E_T^N(30)$ [b]	$\varepsilon_{max\ (MeOH)}$ = 13 [c]			
		λ_{Abs} [d]	λ_{Em} [e]	$\Delta\lambda$ [f]	Φ [g]
EtOH	0.65	528	656	128	1
CH_3CN	0.46	531	654	123	4
DMSO	0.44	544	666	122	4
DMF	0.39	535	635	100	12
DCE	0.33	533	631	98	15
EtOAc	0.23	514	611	97	22
THF	0.21	514	607	93	65
Toluene	0.10	510	584	74	91

[a] Reported values are the average of two or more independent and reproducible measurements, ±1 nm for wavelengths. Excitation wavelength was at the corresponding absorption maximum. [b] Normalized Reichardt's solvent polarity index [52]. [c] Molar extinction coefficient in $10^3\ M^{-1}\cdot cm^{-1}$ was determined in methanol; relative standard deviations are lower or equal to 5 %. [d] Position of the absorption maximum in nm. [e] Wavelength of the emission maximum in nm. [f] For convenience, Stokes shifts are expressed in nm for $\Delta\lambda = \lambda_{Em} - \lambda_{Abs}$ (difference in cm^{-1}). [g] Fluorescence quantum yields Φ were determined using an excitation at the corresponding absorption maximum of the reference standard in the considered solvent: Nile blue perchlorate in EtOH (λ_{Ex} = 321 nm, Φ = 27 %)[53], ±10 % mean standard deviation.

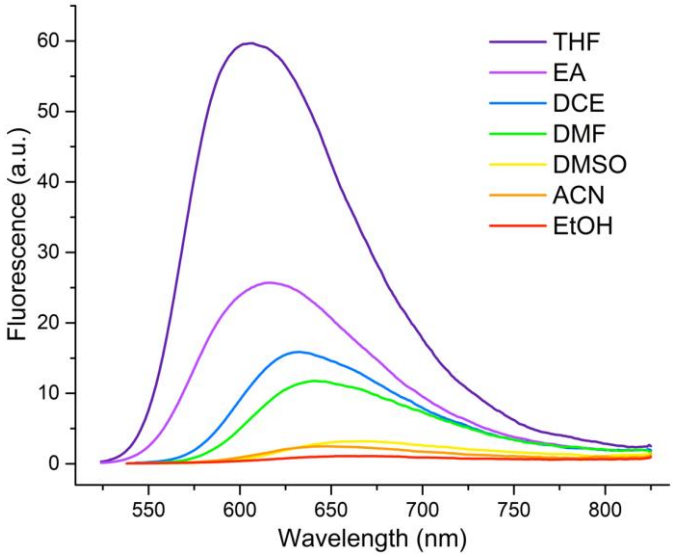


Fig. 6. Emission spectra of the derivatized adduct **CypPyXa**, demonstrating fluorescence quenching over the increasing polarity.

Fluorescence measurements were performed in the same set of solvents (Table 1 & Fig. 6). After selective excitation, **CypPyXa** exhibited significant positive solvatofluorochromism (~ 70 nm) with maxima oscillating from 584 to 656 nm and an emission color varying over *yellow*⇔orange⇔red (Fig. S2). Mega-Stokes shifts (>100 nm) were even observed in polar solvents, attesting to the strong dependance of the excited-state dipole along the increasing polarity; typical of push–pull fluorophores. In parallel, quantum yields fluctuate over the entire range, making the dye extremely bright in toluene (91 %) and strongly quenched in protic medium, like EtOH (1 %). Such a fluorescence quenching – resulting from the steady increase of polarity – is characteristic of a substantial push–pull relationship being established between the pyrrolo donor (D) and the nitro acceptor (A) groups. In terms of solubility, reactivity and brightness, DMF appeared to be the best compromise for this derivatization reaction.

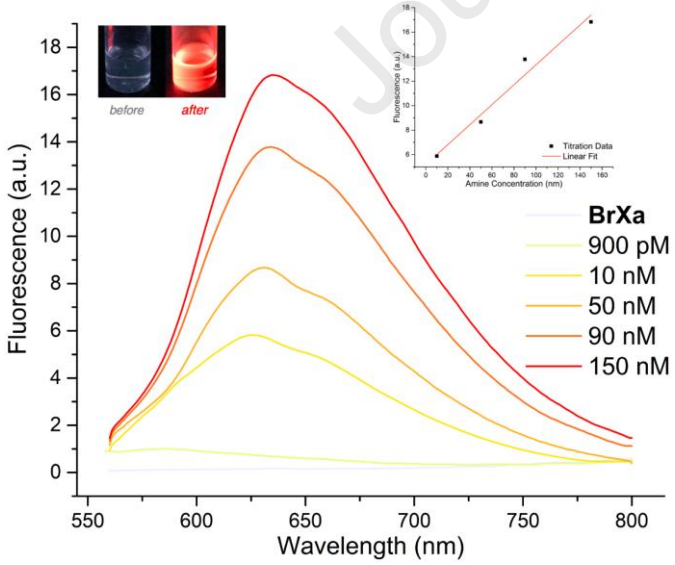


Fig. 7. Fluorogenic sensing of Cyp-NH_2: determination of the sensitivity limit of **CypPyXa** by fluorescence titration of **BrXa**. Inserts represent the starting *(left)* and final *(right)* solutions, respectively non-emissive and strongly red fluorescent.

Lastly, the sensitivity limit was determined in order to define the scope of applicability of this derivatization process. By exciting at the absorption maximum of **CypPyXa** (535 nm), the initial **BrXa** solution proved to be completely "dark" (flat curve and insert, Fig. 7). This is an essential prerequisite for the development of a fluorogenic derivatization method. The increasing addition of Cyp-NH_2 made emanate a bright red emission (*insert* in Fig. 7) resulting from the formation of **CypPyXa**. Plotting the fluorescence intensity as a function of the incremental concentration allowed to estimate that the sensitivity limit was located at the nanomolar scale (*insert* in Fig. 7, Fig. S3). Such a low level of detection combined with the colorimetric and fluorogenic character of this simple procedure, constitute as many assets to promote this derivatization method of primary amines.

To prove the potential applicability of **BrXa** to the study of biological samples, additional reactions were conducted with various thiols. Indeed, this class of compounds is known to include the strongest nucleophiles in biological media. Control experiments were performed with 2-mercaptoethanol and a L-cysteine derivative (Fig. S4–5). As expected, the substitution product was mainly formed but no color and fluorescence appearance was detected (Fig. S6–9). This confirms that the chromogenic and fluorogenic character of this derivatization is directly related to the sensing of 1° amines. Competition reactions between thiol *vs.* amine were also carried out to demonstrate that only a small excess of **BrXa** allows the detection of a primary amine even in the presence of a reactive thiol. Since only the 1° amine derivatization products generate colored and fluorescent products, the slight excess of this "completely transparent" reagent should not interfere with spectrophotometric detection.

3.5. Quantum calculations

Gaussian 09 was employed for calculating the theoretical optical and quantum chemical properties of **BrXa** and the derivatized amino product as well as for determining their geometry in both ground and excited states. To simplify the calculations, methylamine was chosen as the reacting species (**MePyXa**). DFT and TD-DFT were used at the B3LYP/ 6-311+G(d) level of theory for the geometry optimization of both molecules in their ground and excited states, respectively, verified by Hessian analysis. Calculations of the electronic absorption spectra were completed by selecting singlet vertical excitation energies and their oscillator strengths were determined by TD-DFT. It was found that the first excited state would fluoresce, therefore the IROOT=1 keyword was utilized. Fluorescence spectra were calculated at the single point level of the optimized geometry in the excited state.

Figure 8 shows the differentiation of the bond lengths upon excitation. For the reagent, the differentiation is associated with the benzene ring 3, with the largest elongation and de-conjugation of the aromatic ring being apparent for the two nitro group oxygens (Table S1). Similarly, but to a far lesser extent, the carbonyl oxygen partially lost its double bond character. **MePyXa** exhibits a similar trend, but the main geometric changes are expanded to rings 2 and 4, in addition to ring 3. As the structure of this derivative presents more extended π-delocalization and consequently increased rigidity, it should induce larger Stokes shifts and enhanced fluorescence quantum efficiency regarding its emission properties. The nitro group does show an important geometrical alteration in both cases, but to a lesser extent for **BrXa** (Fig. 8). All the dihedral angles of **BrXa** and **MePyXa** are >177°, pointing out high aromaticity, both in the ground and excited states.

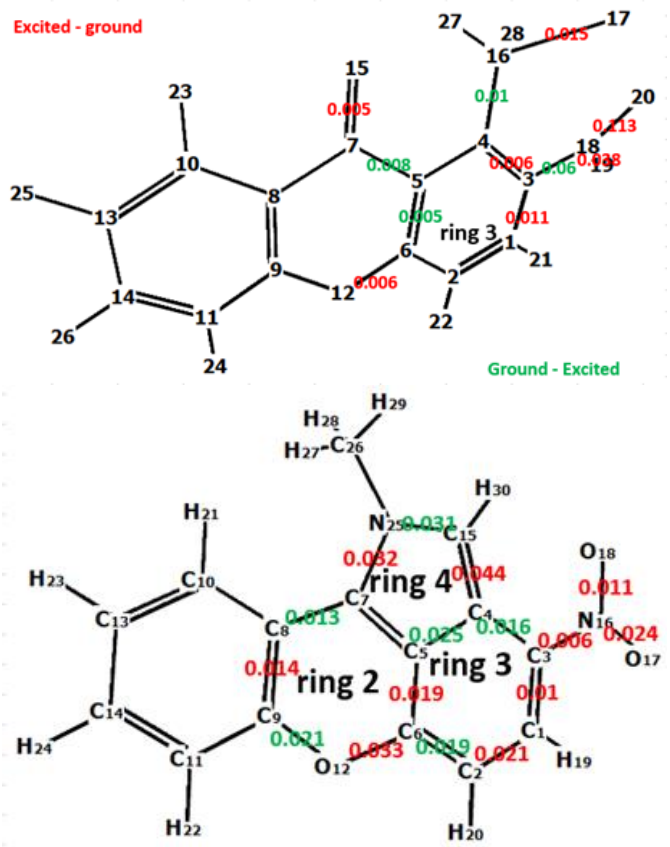


Fig. 8. Bond elongation features upon excitation for **BrXa** *(top)* and **MePyXa,** the methylamine-derivatized product *(bottom). Only bond length differences larger than 0.005 Å were reported.*

The HOMO and LUMO orbitals of **MePyXa** are delocalized, highlighting the aromatic character of the substance. Apparently, the LUMO orbital is more localized on certain atoms compared to the more widespread HOMO. This observation is also reflected in the distribution of the Mulliken charges (Table S2). Since the calculated HOMO–LUMO energy difference turns out to be small (2.76 eV), this indicates that the derivative is highly conjugated. Excitation occurs from HOMO (Fig. 9A) towards LUMO (Fig. 9B), which corresponds to λ = 490 nm affording an oscillator strength of f = 0.31. A second, less intense excitation (f = 0.13) is predicted from HOMO to the second, lower lying LUMO with an energy gap of 3.46 eV, which corresponds to λ = 358 nm. Finally, the dipole moment lies on the long axis of the compound showing a total magnitude of 9.6 D. The emission from the geometry-optimized excitation level between HOMO and LUMO occurs with an orbital state difference of 2.46 eV corresponding to an emission λ = 565 nm with an oscillator strength of f = 0.21 (Fig. 10). Smaller oscillator strength transitions were observed at 359 nm (f = 0.146), 328 nm (f = 0.167) and 316 nm (f = 0.04). The dipole moment was calculated mainly along the x axis, affording a value of 8.8 D, attesting to a significant charge separation in the excited state. Thus, the derivatization product becomes noticeably polarized upon photon absorption, which is characteristic of molecules interacting efficiently with light.

On the other hand, the direction and magnitude of the dipole moment vector reveal that the nitro group is essential for the fluorescence emission by establishing a push–pull relationship with the electron-donating *N*-methylpyrrole. This was verified experimentally by reduction of the nitro group to an amine, yielding derivatives practically devoid of fluorescence. Specifically, this chemical reduction converts initially D-π-A fluorophores into D-π-D, known to exhibit a strongly quenched and blue-shifted emission.

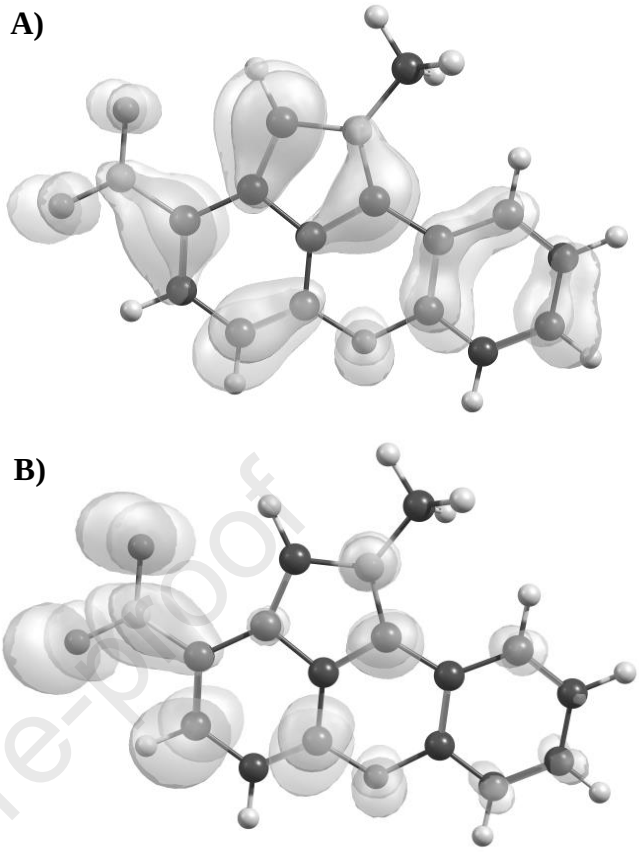


Fig. 9. HOMO (A) and LUMO (B) of the derivatization product of methylamine.

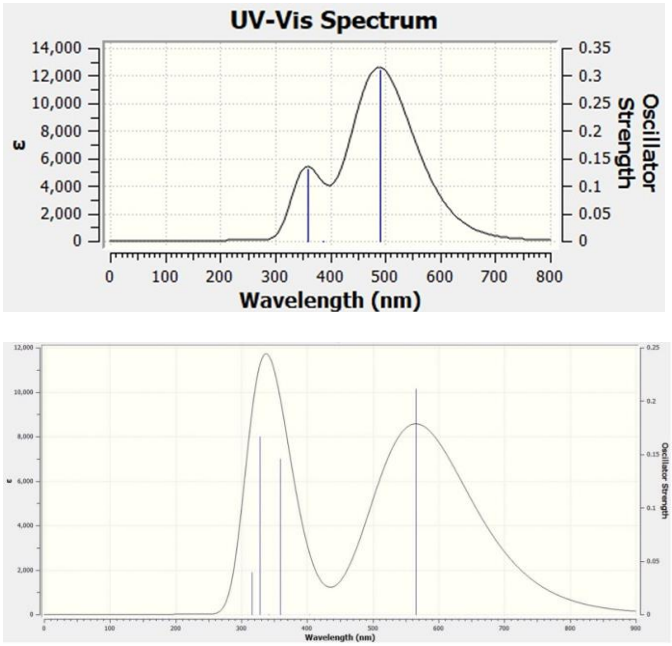


Fig. 10. Theoretical excitation *(top)* and emission *(bottom)* spectra of the derivatized amine.

4. Concluding remarks

Since amines are present in many naturally occurring molecules – including proteins, peptides, nucleic acids, or alkaloids – fishing for this functional group in a complex mixture at low concentrations, represents a major challenge. In this context, a

xanthene-based derivatization reagent **BrXa** was engineered, which combines both colorimetric and fluorogenic sensing, exclusively for primary amines. Its preparation relies on an efficient and scalable synthesis. Given that the detection reaction is based on a pyrrole formation, its selectivity towards primary at the expense of secondary and tertiary amines revealed to be extremely high. As the process is chromogenic (colorless ⇔ magenta), this should interestingly make the method more accessible and enable to reach a wider audience. Besides the appearance of an easily detectable fluorescent color (dark ⇔ red), the fluorogenic nature of this detection turns out to be a real asset. As a matter of fact, it allows to benefit from the exquisite sensitivity of the fluorescence spectroscopy, making it possible to achieve the nanomolar range. Supported by quantum calculations and photophysical characterization, the push–pull relationship from which the red fluorescence originates, was clearly evidenced. Combining all these attractive features with the development of a robust LC analytical method, offers a unique turnkey solution for the derivatization of primary amines.

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Appendix A. Supplementary data (Material)

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dyepig.2021.x.x.

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DYES
& Pigments

HIGHLIGHTS

The identification of challenging amines in complex extracts of natural products requires more and more innovative and sensitive spectroscopic methods.

- A xanthene-based derivatization reagent was engineered for the exclusive detection of primary amines.
- Thanks to its chromogenic nature (colorless ⇔ magenta), the derivatization process becomes accessible to a broader audience.
- The developed fluorogenic sensing (non-fluorescent ⇔ red) allows the method to reach an exquisite level of sensitivity, approaching the picomolar range.

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: