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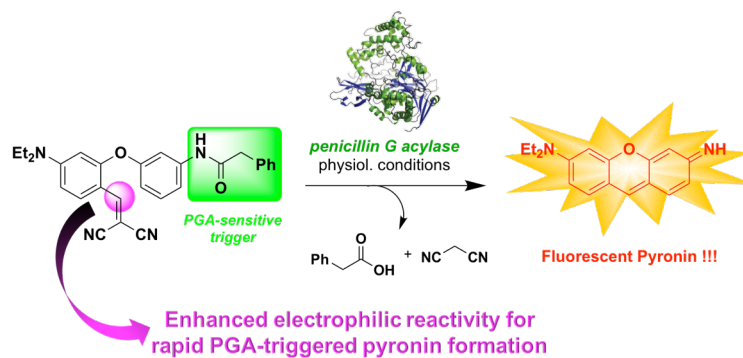
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Kinetics improvement of protease-mediated formation of pyronin dyes

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Kinetics improvement of protease-mediated formation of pyronin dyes

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ABSTRACT

A fluorescent probe for protease sensing and based on the "covalent-assembly" principle is reported. The basic rationale for this unusual class of chemodosimeters proposed by the Anslyn and Yang groups entails the synthesis of non-fluorophore caged precursors full-stable and reactive towards the targeted analyte. Unlike the first generation of protease-sensitive "covalent-assembly" type probes recently published by ourselves (*Org. Biomol. Chem.* **2017**, *15*, 2575-2584), the availability of dicyanomethylidene and enzyme-labile phenylacetamide moieties within the core structure of mixed bis-aryl ether **2** enables its rapid conversion into a fluorescent pyronin dye at physiological pH and upon activation with penicillin G acylase (PGA). This is real progress towards the practical implementation of this ingenious activation mechanism to the detection of enzymes in their native environment (*in cellulo* or *in vivo*).

1. Introduction

Over the past two decades, activatable (or "smart") fluorescent probes have become established for bioanalytical landscape and more recently in the fields of molecular imaging and theranostics.¹⁻³ The vast majority are designed on the basis of well-known photophysical processes (*e.g.*, self-quenching and exciton coupling, resonance energy transfer, PeT, ...) and/or fluorogenic reactions that often involve transformation of a functional group acting as a fluorescence switch (typically, bond-cleavage reactions, organic addition and/or metal-ligand substitution reactions and cascade reactions).^{1,5} These advanced chemical tools enable the detection of analytes-of-interest with good to high sensitivity and unrivaled spatiotemporal resolution, in the context of complex biological or environmental matrices.

To overcome some limitations of these probes and to expand their scope of application, a new probe design principle namely the "covalent-assembly" approach was recently proposed by Anslyn and Yang.^{6,7} The basic rationale of the "covalent-assembly" type probes is the *in situ* formation of the electronic push-pull conjugated backbone of a fluorescent organic dye, from a non-fluorophore caged precursor and *via* a cascade reaction triggered by the analyte-of-interest. The main advantage of this approach is to produce reaction-based fluorescent probes (also known as fluorescent chemodosimeters or pro-fluorophores) with zero background signal and hence particularly useful for applications that require high detection sensitivity. Its practical utility in molecular sensing is highlighted by the large number of recent publications dealing with the detection of various analytes (*i.e.*, biothiols, enzymes, metal cations, nerve agents and ROS/RNS

through *in situ* formation of fluorescent 7-*N,N*-dialkylamino- or 7-hydroxy-(2-imino)coumarin or pyronin/rosamine scaffolds.^{6,8,9} Further extension of the "covalent-assembly" principle to other less popular fluorescent molecules (*e.g.*, benzo[*c*]cinnoline¹⁰, benzotriazole¹¹, coumarin-fused resorufin¹², diazachrysenes¹³, phenanthridine¹⁴ and pyrazino-benz[*e*]indole derivatives¹⁵) was also reported but the cascade reaction triggered by the analyte-of-interest is often related to the specific reactivity of the latter and therefore not suitable for developing a versatile molecular chemosensing approach. Finally, some pioneering works have demonstrated that fluorescence biosensing at longer wavelengths (in the orange-red, far-red or near-infrared (NIR) spectral range) can be readily achieved through internal construction of polymethine dyes.^{16,17} Indeed, biocompatible addition-elimination reactions leading to the formation of azomethine or dimethine bridges found in (hemi)cyanine scaffolds, have already been used to illuminate biological structures *in vitro* or in living cells (*e.g.*, DNA G-quadruplexes^{16b} and cellular retinoic acid binding protein II (CRABP II)¹⁷).

To implement the promising concept of "covalent assembly" to *in vivo* molecular imaging of disease-related enzymes¹⁸, a first step was taken by our group with the clearly and indisputable demonstration of *in situ* formation of a yellow-orange emitting pyronin fluorophore **1**, from the mixed bis-aryl ether **2** as caged precursor (Fig. 1), triggered by a protease (*i.e.*, penicillin G acylase (PGA) or leucine aminopeptidase (LAP) enzyme).¹⁹ However, the rate of cyclization/aromatization process was found to be slow and the use of an acidic buffer solution or additives known to activate

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the carbonyl moiety of **2** (e.g., ammonium sulfate), was required to obtain its quantitative conversion into pyronin **1** (6-*N,N*-diethylamino-3*H*-xanthen-3-imine) within 24 h of incubation with enzyme. To overcome this major shortcoming, particularly in view of implementing our small molecule detection platform for protease sensing in living biological systems (*i.e.*, living cells and small animals), further structural optimization of this unusual type of fluorogenic enzyme substrates was considered.

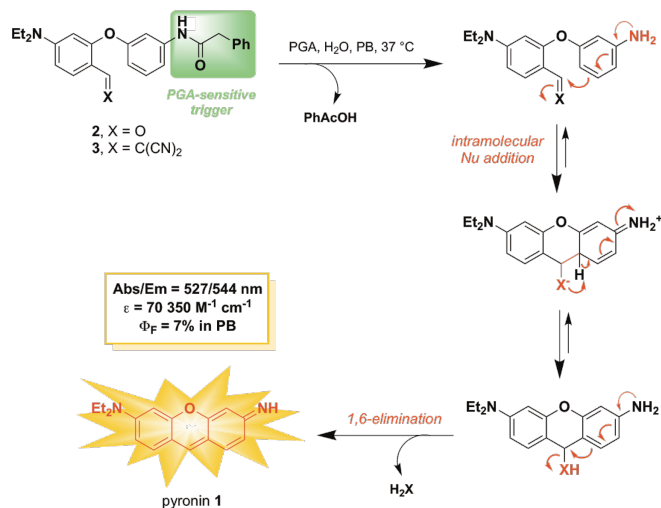


Figure 1. Proposed detection mechanism of proteases (PGA chosen as model enzyme, also known as penicillin amidase, EC 3.5.1.11) based on the "covalent-assembly" principle and the use of caged precursors **2** and **3** convertible into fluorescent pyronin **1**.

In this Letter, we report the valuable findings from this survey through the comprehensive description of synthesis, photophysical characterization, fluorogenic behavior and enzymatic activation of a novel PGA-sensitive caged precursor **3** rapidly converted to pyronin **1** under physiological conditions.

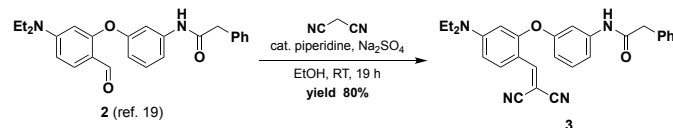
2. Results and discussion

The rationale behind a potentially profitable structural optimization of a pyronin caged precursor is to find the right balance between its reactivity (*i.e.*, effectiveness of enzyme-triggered cascade reaction) and its stability (*i.e.*, inertness of the probe in the absence of the targeted enzyme). Starting from the core structure **2**, the most obvious way to achieve this goal is to subtly enhance the electrophilic character of the aldehyde undergoing an intramolecular nucleophilic addition of the adjacent phenylogous amine unit unveiled by enzymatic hydrolysis. We thought to convert the formyl group into dicyanomethylidene moiety, whose electron-withdrawing properties are often exploited to design push-pull fluorophores with strong intramolecular charge transfer (ICT) character.²⁰ The presence of electron-donating *N,N*-diethylamino group in *para* position should confine the addition of water to this Michael acceptor leading to undesired hydrolysis of this benzylidenemalonitrile derivative.²¹ Also, since the malonitrile anion is a better leaving group than the hydroxyde anion (pKa malonitrile = 11), a positive effect on the rate of 1,6-elimination process contributing to xanthen aromatization, could also be expected.

Synthesis of dicyanomethylidene-based caged precursor **3**

As previously reported by us¹⁹, the PGA-sensitive probe **2** was readily synthesized in two steps from commercial 3-iodoaniline (*i.e.*, *N*-acylation with phenylacetyl chloride followed by Ullman-type coupling with 4-(*N,N*-diethylamino)salicylaldehyde). Its conversion into dicyanomethylidene derivative **3** was achieved by

reaction with malonitrile under mild conditions already optimized by our group for the synthesis of dual enzyme-responsive caged precursors of 7-hydroxy-(2-imino)coumarins: cat. piperidine, anhydrous Na₂SO₄, in EtOH and at room temperature (Scheme 1). Purification by conventional column chromatography over silica gel provided the dicyanomethylidene-based caged precursor **3** in a good 80% yield. All spectroscopic data (see Supplementary data), especially IR, NMR and mass spectrometry, were in agreement with the structure assigned. Its high level of purity was confirmed by RP-HPLC analysis and found to be in the range 96-99% depending the wavelength used for UV-visible detection.



Scheme 1. Synthesis of PGA-sensitive fluorogenic probe **3**.

Photophysical characterization of dicyanomethylene-based caged precursor **3**

UV-visible and fluorescent measurements were conducted in phosphate buffer (PB, 100 mM, pH 7.6, simulated physiological conditions) containing less than 0.25% of DMSO (originating from dilution of 1.0 mg/mL stock solution in this latter solvent), and the corresponding spectral curves are given in Fig. 2. The PGA-sensitive probe **3** exhibits a strong electronic absorption in the violet-blue range (Abs λ_{max} = 467 nm and shoulder at λ = 440 nm with ϵ = 31 220 M⁻¹ cm⁻¹ and 27 770 M⁻¹ cm⁻¹ respectively) characteristic for an ICT transition from the *N,N*-diethylamino donor group to the dicyanomethylidene acceptor fragment. A further evidence of this is the large value of spectrum width (full-width half maximum, $\Delta\lambda_{1/2 \text{ max}}$ = 90 nm). Contrary to the trend noted for aldehyde-based caged precursor **2**, excitation at 440 nm (465 nm, or 485 nm), leads to a strongly Stokes-shifted ICT emission band centered at ca. 600 nm (fluorescence quantum yield Φ_{F} = 6% determined using Ru(bpy)₃Cl₂ as standard (Φ_{F} = 4.2% in water)²²). Further investigation following up an unexpected result from HPLC-fluorescence analyses of enzymatic reaction mixtures (*vide infra*) has shown the complete loss of fluorescence properties in organic media (especially in CH₃CN, see Fig. S1). This photophysical behavior is typical of molecules that exhibit aggregation-induced emission (AIE) behavior, and known as AIEgens.²³ Moreover, it is worthwhile mentioning that among the structural motifs found in AIEgens, the dicyanovinyl group is often used to decorate the popular tetraphenylethene (TPE) luminogen whose the AIE activity is ascribed to restricted intramolecular rotation (RIR) in aq. environment. The formation of such aggregates in PB is further supported by the recording of excitation spectrum of **3** that does not perfectly match with its absorption curve (Fig. 2). The main consequence of this lack of a zero background signal for the caged precursor **3** is that the detection of enzyme activity should be achieved through a ratiometric and not intensometric fluorogenic response as initially hoped.^{2,4} Fortunately, the absorption ability of **3** at 525 nm is weak (Ex wavelength used for fluorescence-based *in vitro* assays) and its fluorescence emission at 545 nm (Em λ_{max} of pyronin **1** formed *in situ*) is almost negligible whatever the excitation wavelength used (440 nm, 465 nm or 485 nm) (Fig. 2 and 4).

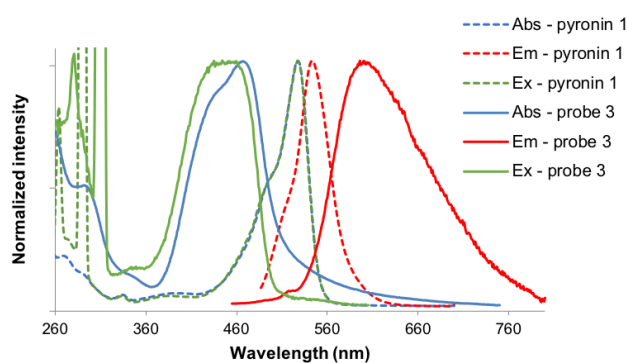


Figure 2. Normalized absorption (blue), excitation (Em. 580 or 620 nm, slit 5 nm, green) and emission (Ex. 440 or 470 nm, slit 5 nm, red) spectra of pyronin **1** and dicyanomethylidene-based caged precursor **3** in PBS at 25 °C. Please note: for excitation spectrum, intense peak at 290 or 310 nm ($\lambda_{Ex}/2$) is assigned to Rayleigh scattering.

Comparative enzymatic activation of "turn-on" fluorogenic probe **2** and ratiometric fluorogenic probe **3**

Fluorescence-based *in vitro* assays were performed with commercial PGA (from *Escherichia coli*) through time-course measurements. The resulting kinetic curves are shown in Fig. 3. A rapid and gradual increase of fluorescence emission at 545 nm (Ex at 525 nm) was observed when dicyanomethylidene-based probe **3** was incubated with PGA at 37 °C and the plateau (meaning that

3 is quantitatively converted into pyronin **1**) was reached within 1 h (for the corresponding time-dependant emission spectra, see Supplementary data). Conversely, when the parent aldehyde-based probe **2** was reacted with PGA under the same conditions, the level of fluorescence achieved is dramatically lower (225 AFU vs 9630 AFU, within 1 h) confirming the slow formation of the xanthene-based fluorophore. This valuable result confirms our hypothesis that the rate of cyclization/aromatization process can be readily promoted by enhancing (1) the electrophilic character of reactive position undergoing intramolecular nucleophilic addition of adjacent phenylogous amine, and (2) the rate of 1,6-elimination of C-9 substituent. Interestingly, a direct comparison of the fluorescence emission spectra of PGA-sensitive probe **3** recorded before and after incubation with enzyme (Ex at 440 nm and 485 nm, Fig. 4), highlighted a spectral blue-shift of 50 nm allowing a proper ratiometric detection. Further kinetics in two other aq. buffers at acidic and basic pH respectively (acetate buffer, 100 mM, pH 5.6 and borate buffer, 100 mM, pH 8.6) were performed (see Fig. S2). In contrast to that observed with aldehyde-based probe **2**, acidic pH provided a significant lower fluorogenic response compared to that observed at physiological pH (6350 AFU vs 11240 AFU after 85 min of incubation). Indeed, dicyanomethylidene moiety cannot be further activated by protonation unlike the carbonyl group. Finally, blank experiments without PGA were performed and have clearly shown the full stability of both probes. Indeed, no fluorescence signal changes were observed in the absence of this amidase.

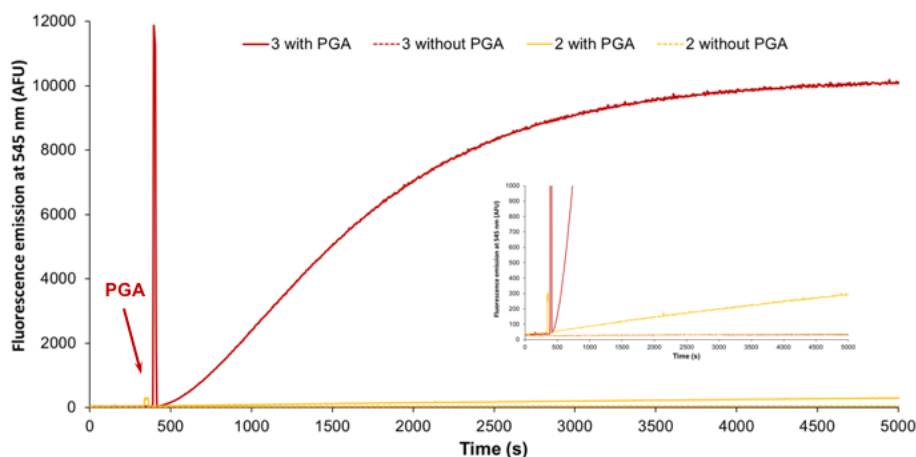


Figure 3. Time-dependant changes in the green-yellow fluorescence intensity (Ex/Em 525/545 nm, slit 5 nm) of fluorogenic probes **2** and **3** (concentration: 1.0 μ M) in the presence of PGA (1 U) in PB (100 mM, pH 7.6) at 37 °C. Please note: PGA was added after 5 min of incubation of probe in PB alone.

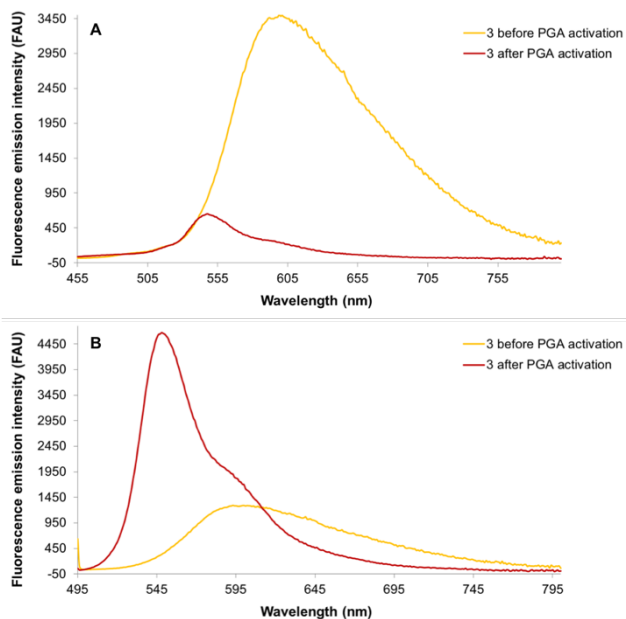


Figure 4. Fluorescence emission spectra of fluorogenic probe **3** before and after incubation with PGA (1 U, 240 min of incubation in PB at 37 °C). (A) Ex at 440 nm (slit 5 nm) and (B) Ex at 485 nm (slit 5 nm).

The presence of pyronin dye **1** in these enzymatic reaction mixtures was unambiguously confirmed by RP-HPLC analyses (fluorescence detection, $t_R = 4.0$ min) and compared with an authentic sample of synthetic pyronin **1** used as reference (Fig. 5). The unexpected lack of a peak for the starting probe **3** on the RP-HPLC elution profile with a fluorescence detection channel Ex/Em 440/600 nm, strongly supports the AIE character of this dye molecule. Indeed, its elution from the C₁₈ column was observed at ca. 100% CH₃CN ($t_R = 6.0$ min), a solvent in which the formation of fluorescent aggregates is not observed (*vide supra*).

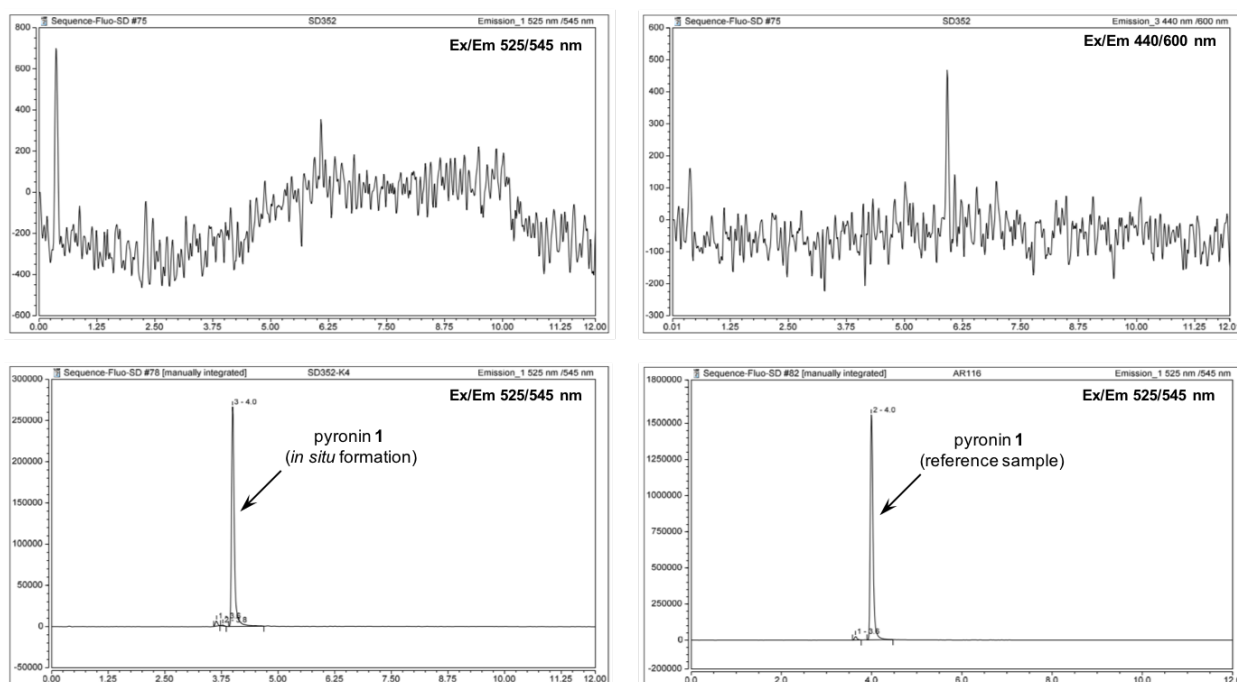


Figure 5. RP-HPLC elution profiles (fluorescence detection, system B) of fluorogenic probe **3** before incubation with PGA (top) and after incubation with PGA (1 U, 250 min of incubation in PB at 37 °C, bottom left), and authentic sample of pyronin **1** (bottom right). Please note: for RP-HPLC elution profile of blank (fluorogenic probe **3** incubated in PB alone) see Fig. S3.

Conclusion

In summary, we have found a clever and easily implementable solution to dramatically increase kinetic of a biocompatible reaction working under physiological conditions, triggered by a protease and leading to *in situ* construction of fluorescent pyronin dyes. This is a major achievement since it will help to apply the "covalent-assembly" probe design principle for interrogating various fundamental biological questions through biosensing/bioimaging operations. We are aware that the systemic toxicity of malonitrile (*i.e.*, metabolic release of cyanide ions) might prevent the use of such dicyanomethylidene-based probes in living systems. Thus, other Michael acceptor adducts derived from easily enolizable Meldrum's acid or 1,3-dimethylbarbituric acid should be considered. More generally, a subtle balance between stability and reactivity of pyronin caged precursors depending on the matrix to be analyzed and targeted application, can be achieved by gradual increasing of the electrophilicity of carbon center undergoing intramolecular nucleophilic addition of adjacent phenylogous amine (*i.e.*, formyl, α -trifluoromethyl carbonyl and dicyanomethylidene moiety). In addition to these valuable findings related to the rational design of assembly type probes, we have identified a novel red-emissive fluorophore scaffold with AIE characteristics. Further works aimed at converting this luminogen into useful biological probes are in progress and will be reported in due course. Lastly, the presented enzyme-triggered cyclization/aromatization reaction could be a useful source of inspiration to organic chemists working in the field of total synthesis of natural products bearing a xanthene scaffold, especially through the development of effective biomimetic cascades.²⁴

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Supplementary data

Supplementary data (synthetic procedure and spectroscopic characterizations of compound **3**, *in vitro* fluorescence assays and HPLC-fluorescence analyses described in this work) associated with this article can be found, in the online version:

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