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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 rational 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 dicyanomethylidenyl 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*).

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, ...)[2,4] 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 rational 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*]cinnoline,[10] benzotriazole,[11] coumarin-fused resorufin,[12] diazachrysene,[13] phenantridine[14] and pyrazino-benz[*e*]indole derivatives[15]) 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 (CRABPII)[17]).

To implement the promising concept of "covalent assembly" to *in vivo* molecular imaging of disease-related enzymes[18], a first step was taken by our group with the clearly and indisputable demonstration of *in situ* formation of a yellow-orange emitting pyronin

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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 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.

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.

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 dicyanomethylidenyl 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 xanthene aromatization, could also be expected.

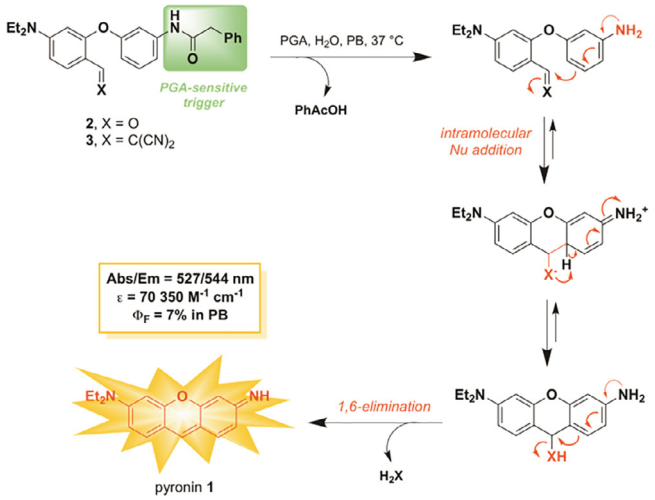


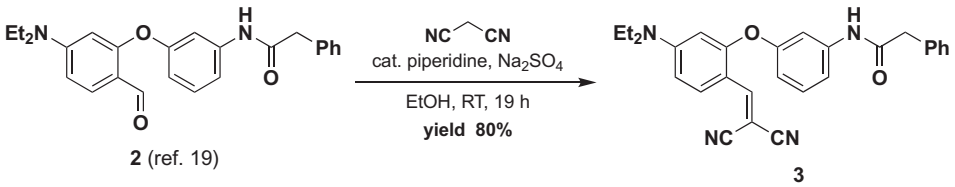
Fig. 1. Proposed detection mechanism of proteases (PGA chosen as model enzyme, also known as penicillin amidase, EC 3.5.1.11) based on the "covalent-assembly" principle and the use of caged precursors **2** and **3** convertible into fluorescent pyronin **1**.

Synthesis of dicyanomethylidene-based caged precursor 3

As previously reported by us[19], 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)salicaldehyde). 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-imono)coumarins: cat. piperidine, anhydrous Na_2SO_4, 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.

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 = 31220 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 dicyanomethylidenyl acceptor fragment. A further evidence of this is the large value of spectrum width (full-width half maximum, $\Delta\lambda_{1/2max}$ = 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 $Ru(bpy)_3Cl_2$ 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 CH_3CN, 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



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