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# Latent fluorophores based on a Mannich cyclisation trigger

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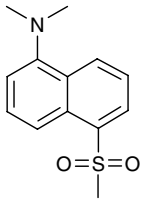
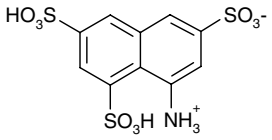
**Abstract**—We report the synthesis of a strongly blue-fluorescent pyrazino-benz[*e*]indole derivative, and its utility for enzyme sensing in biological assays through an original Mannich cyclisation triggered fluorescence unveiling.


Latent fluorophores (or pro-fluorophores) are stable probes that unmask their intense fluorescence only by a user-designated chemical reaction. They are especially useful tools for basic research in the biological sciences.[1] Contrary to other fluorescence based probes, those latent fluorophores display a unique selectivity and limited interferences associated with the probe concentration, excitation intensity and emission sensitivity. Thus increasing interest and efforts have been devoted lately to the development of such latent fluorophores. Sophisticated molecules that release a fluorescent coumarine, rhodamine or benzo[*a*]phenoxazine derivative upon activation by an enzyme or reactive oxygen species have been developed for detecting hydrolytic enzyme activities (esterase, protease, epoxide hydrolase, phosphatase, etc.) in biological environments,[2–8] or imaging cellular metabolites.[9–11] Furthermore, new fluorogenic dyes having longer excitation and emission wavelength maxima (in the far-red and near-infrared ranges) are expected to be powerful tools for in vivo imaging applications.[8,12] In this letter, we report our first results towards such a protease detection probe. The aim is to use a non fluorescent amide which, upon protease unveiling of the free amine will yield a fluorescent product through an original approach based on an intramolecular Mannich cyclisation reaction.[13] In this first approach, as a proof of concept, we used a new aminonaphthalene derived short wavelength fluorophore since, among the multitude of available UV light-excitable dyes, aminonaphthalene derivatives exhibit fluorescence properties, which are often sensitive to the environment. Some of them such as Dansyl **1** and ANTS **2** are currently used as cell tracers or tools for protein structural studies.[14]




**1**, Dansyl




**2**, ANTS

Furthermore, it had previously been demonstrated that the quaternisation or protonation of the amino group on substituted naphthalene derivatives resulted in a dramatic reduction in fluorescence intensity.[15,16] We thus selected alkylated 1,1,2-trimethylbenz[*e*]indole derivatives such as iminium salt **3** as the pro-fluorophores, since they should be poorly fluorescent. We then foresaw that the hydrolysis of the carboxamide should generate the free amine **4**, which will spontaneously cyclise by an intramolecular reaction onto the iminium moiety, to give the highly fluorescent pyrazino-benz[*e*]indole derivative **5** (Fig. 1). To our knowledge, this heterocyclic compound has never been described to date. Only the synthesis of compounds **6** and **7** which exhibit some structural similarities with **5** was reported during the last

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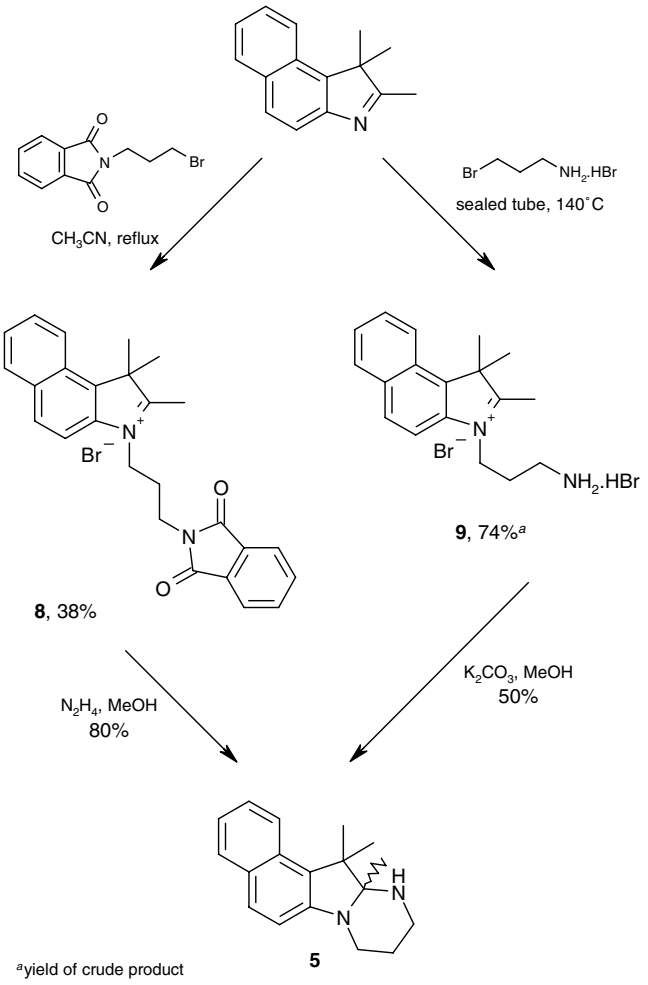
30 years.[17,18] However, no data about their spectroscopic properties are available.

With the goal in mind to develop this new class of efficient and structurally simple fluorophores suitable for various biological applications, we have first explored the synthesis of **5** and studied its fluorescence properties in detail. The pyrazino-benz[*e*]indole derivative was prepared via two different convenient two-step procedures. As shown in Scheme 1, the starting material 1,1,2-trimethyl-1*H*-benz[*e*]indole[19,20] undergoes quaternisation with either (3-bromopropyl)phthalimide or 3-bromopropylamine hydrobromide to give the iminium quaternary salts **8** and **9**, respectively.[21,22] As expected, those two iminium salts did not display significant fluorescence properties. Removal of the phthalimide protective group of **8** was achieved by treatment with hydrazine monohydrate in methanol. Under these neutral conditions, the iminium ion was readily trapped by the released primary amino group to give the fluorophore **5** which was isolated in good yield by silica gel chromatography. Interestingly, this intramolecular Mannich reaction also occurred when quaternary salt **9** was treated with a slight excess of $K_2CO_3$ in methanol. The structure of **5** was confirmed by detailed measurements, including MALDI-TOF mass spectrometry and NMR analyses.[23] As expected, this intramolecular addition was found to be subject to Baldwin's rules concerning the cyclisation aptitude of trigonal systems.[24] Indeed, the same reaction conditions applied to the aminoethyl derivative **10** led to a 7:3 mixture of iminium salt **11** and diazabicyclic compound **12** (Scheme 2). This result corroborates the empirical rule namely the 5-*endo*-trig ring closure is less favoured than the 6-*endo*-trig process leading to the quantitative formation of **5**.




**Scheme 1.** Synthesis of the new fluorophore.

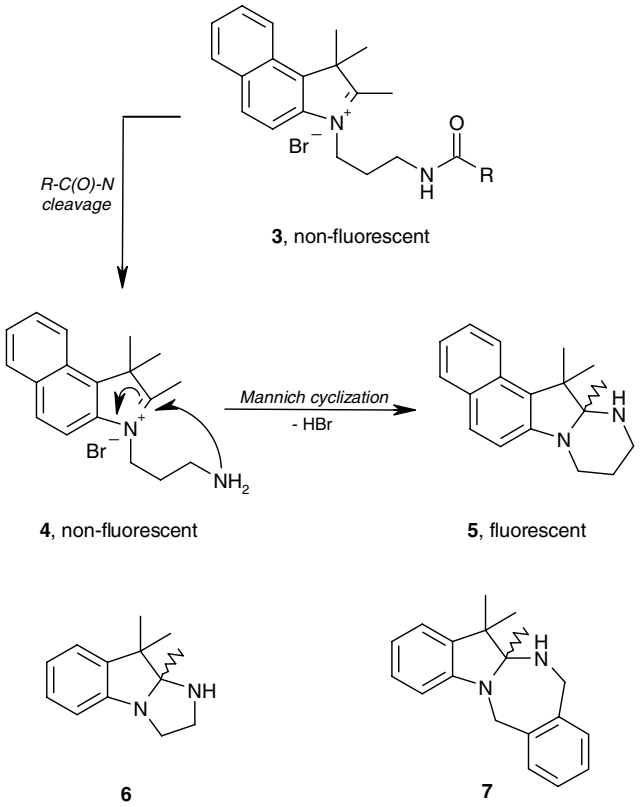
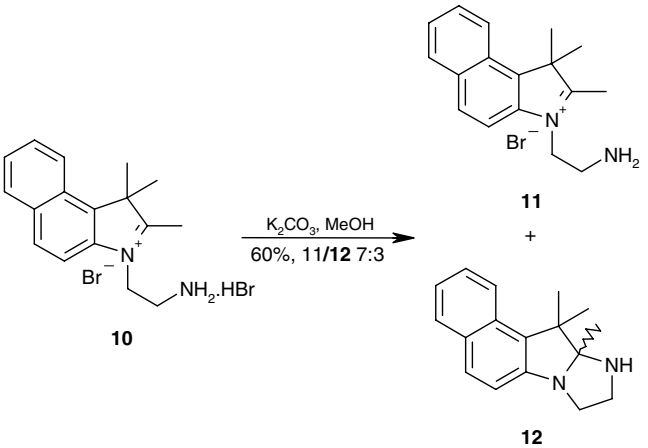



**Figure 1.** Principle of a pro-fluorophore unmasked by Mannich cyclisation and structures of analogues of **5** already reported in the literature.




**Scheme 2.** Mannich cyclisation of aminoethyl derivative **10**.

**Table 1.** Spectral properties of **5** in three solvents

| Solvent | $\lambda_{max,abs}$ (nm) | $\lambda_{max,em}$ (nm) | Stokes shift (nm) | $\varepsilon$ ($dm^3\ mol^{-1}\ cm^{-1}$) | Relative QY[a] |
|---|---|---|---|---|---|
| Phosphate buffer | 245 | 455 | 210 | 28,157 | 0.95 |
| EtOH | 253 | 435 | 182 | 27,864 | 0.25 |
| Aqueous TFA 0.1% | 266, 276 | [b] | [b] | 10,309, 10,123 | [b] |

[a] Determined at 25 °C by using DL-tryptophan in phosphate buffer (QY = 0.12, according to PhotochemCAD database available free of charge via the internet at http://omlc.ogi.edu/spectra/) or anthracene in EtOH (QY = 0.27, according to Ref. 29) as standard.
[b] No detectable fluorescence under these acidic conditions.

Absorption and fluorescence properties for the pyrazino-benz[*e*]indole derivative **5** in phosphate buffer, aqueous acidic trifluoroacetic acid and ethanol are summarised in Table 1. The absorption and emission spectra of **5** in phosphate buffer, as typical examples, are displayed in Figure 2. Under the simulated physiological conditions, this short-wavelength fluorophore exhibits a strong blue fluorescence with an emission maximum around 450 nm and, noteworthingly, a large Stokes' shift (210 nm). Moreover, a particularly high quantum yield was obtained, which could be ascribed mainly to two factors: firstly the strong rigidity of the tetracyclic system, and secondly the efficient rehybridization through an intramolecular charge transfer (RICT) process in the singlet excited state ($S_1$) directed from the N-1 of the pyrazinyl moiety to the naphthalene ring.[†] The observed sensitivity of fluorescence of **5** towards solvent polarity is also consistent with emission from a polar $S_1$ intermediate resulting from this charge transfer process.[25,26] Furthermore, as a first encouraging result for the latent fluorescent unveiling strategy, under acidic conditions, **5** did not produce significant fluorescence.[23] Indeed, protonation at N-3 favours the pyrazinyl ring opening leading to the formation of a nonfluorescent iminium salt.[‡] The disappearance of the low-energy emission band for this latter compound was explained by the lack of an enlarged electronic delocalisation within the benzindoleninium moiety.

Moreover, since **5** displayed its highest fluorescence efficiency in simulated physiological medium, it makes this new fluorophore a perfect tool for enzyme activities sensing. We thus next investigated the potential biological utility of fluorophore **5**. With this goal in mind, and as a first example, we prepared a new fluorogenic substrate of penicillin amidase (penicillin G acylase, PA), **15**. PA is the key enzyme in the industrial manufacturing of semisynthetic penicillins. The enzyme catalyses the hydrolysis of a number of structurally diverse amides of the general form R–CO–NH–R′, where R′ can be varied substantially. At the acyl position (R–CO), however, phenylacetyl is the superior residue.[27] PA was chosen since this enzyme is readily available under an immobilised form which tolerates the use of an organic co-solvent such as acetone or methanol,[28] essential to solubilise pro-fluorophore **15** in enzymatic assays performed with high concentrations (~1 mM) in a fluorogenic substrate.[§] However, **15** was found to be perfectly soluble under simulated physiological conditions in the concentration range (1–50 μM) suitable for fluorometric or colorimetric enzymatic assays. Since the selective enzymatic deprotection of the phenylacetamide group of **15** generates the free primary amine **4**, we expected that it will spontaneously cyclise to give the fluorescent pyrazino-benz[*e*]indole derivative **5**. Scheme 3 outlines the preparation of **15**. Acylation of 3-bromopropylamine with phenylacetyl chloride affords a 9:1 mixture of phenylacetamide derivative **13** and oxazinium salt **14** which was used in the subsequent reaction without further purification. Quaternisation reaction of 1,1,2-trimethyl-1*H*-benz[*e*]indole with **13/14** proceeds smoothly to generate the targeted fluorogenic substrate **15** in 50% yield after work up and purification by column chromatography.

As expected, this compound was found to be nonfluorescent especially in phosphate buffer.[23] It was then assayed with its target amidase, immobilised PA. Figure 3 showed the fluorescence emission time course for the enzyme catalysed hydrolysis.[¶] After addition of PA to the substrate solution, a strong fluorescent signal generated at 455 nm indicated the catalytic cleavage of the amide bond and release of free amine which spontaneously cyclised yielding highly fluorescent tetracyclic derivative **5**. Furthermore, no nonspecific cleavage of the probe was detected in a control reaction where **15** was incubated only with the PA buffer. Further evidence of the selectivity of the PA-initiated cleavage was provided by RP-HPLC analysis of the crude reaction mixture of an independent enzymatic assay performed at a larger scale. Indeed, after 4 h of incubation, a major peak corresponding to the pyrazino-benz[*e*]indole derivative **5** was observed (yield of **5** estimated at 95%).[23]

In conclusion, we have presented the first synthesis and fluorescence characteristics of an original pyrazino-benz[*e*]indole derivative. Preliminary experiments with a model enzyme have clearly shown that Mannich cyclisation-triggered latent fluorophores releasing this deriva-

[†] RICT process is promoted by the enlarged electronic delocalisation caused by the conjugation of the lone pair of N-1 atom with π electrons of the naphthalene ring.
[‡] Conversion into the iminium salt was confirmed by RP-HPLC analyses and after co-injection with a standard independently prepared from 1,1,2-trimethyl-1*H*-benz[*e*]indole and 3-bromopropylamine hydrobromide. Its structure is similar to that of compound **9**, except for the nature of counter-ion (TFA instead of $Br^-$).

[§] This was the case for our enzymatic assays followed by RP-HPLC and performed at a concentration of 2.7 mM.
[¶] The use of a nonsoluble biocatalyst and the absence of a continuous stirring (to avoid fluorescence signal disturbances) explain that this enzymatic reaction has taken a long time (24 h) to go to almost completion.

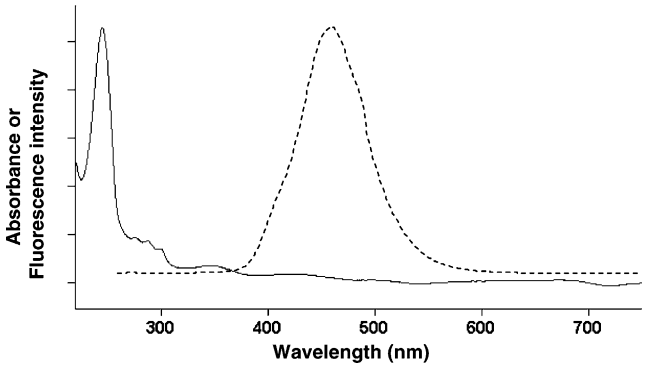
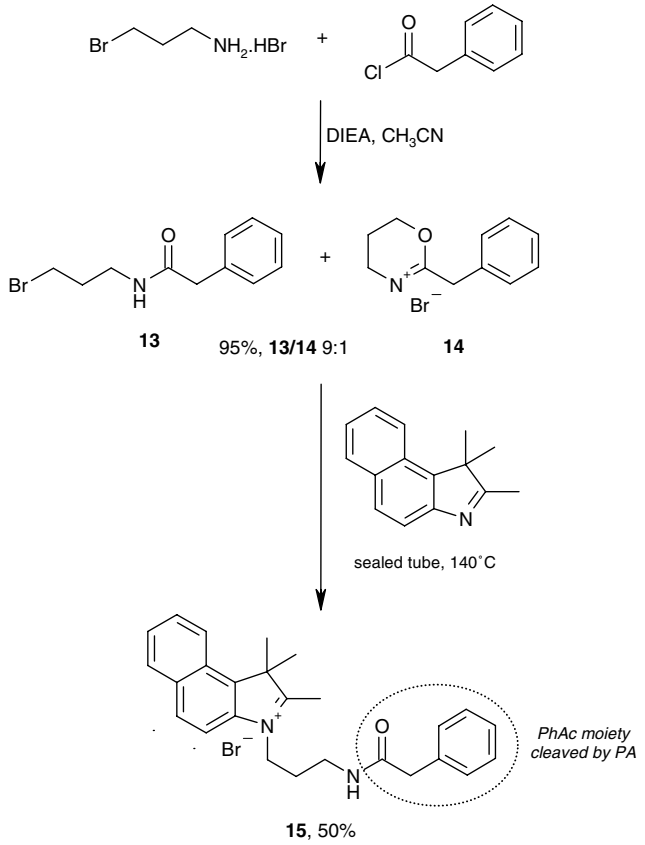



**Figure 2.** Normalised absorption and emission spectra of **5** in phosphate buffer at 25 °C.




**Scheme 3.** Synthesis of fluorogenic substrate of PA.

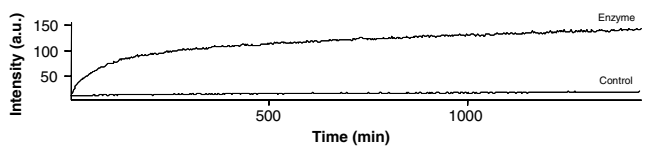



**Figure 3.** Fluorescence assay for the hydrolysis of **15** (1 μM) with immobilised PA (6.1 U) in phosphate buffer (50 mM, pH 7.5). The fluorescence excitation and emission were 240 and 455 nm, respectively.

tive could be used as proteolytic enzyme sensors (detection and quantification) in biological assays. Further efforts are in progress to improve the water solubility of this UV light-excitable dye and to shift its absorption maximum to complex proteic media compatible wavelengths (~350 nm) especially by introducing sulfonate groups onto the naphthalene ring. Thus, it will be possible to get efficient substitutes of short-wavelength fluorophores commercially available from Invitrogen such as Alexa Fluor® 350 and Marina Blue® dyes.[14]

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## Supplementary data

Detailed synthetic procedures, spectroscopic and photophysical characterisations of **5** and **15**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2006.06.138.

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