Organic & Biomolecular Chemistry

Accepted Manuscript

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Organic & Biomolecular Chemistry

PAPER

Dual enzyme-responsive "turn-on" fluorescence sensing systems based on *in situ* formation of 7-hydroxy-2-iminocoumarin scaffolds†

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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A new strategy for the simultaneous fluorogenic detection of two distinct enzyme activities namely hydrolase (amidase or esterase) and reductase is described. This innovative biosensing method is based on the powerful "covalent-assembly" principle that involves *in situ* synthesis of a fluorophore from a non-fluorescent caged precursor and through domino reactions triggered by the two analytes of interest. To establish this approach, penicillin G acylase (PGA) (or pig liver esterase (PLE)) and nitroreductase (NTR) were chosen as model enzymes, and original bis-*O*-protected 2,4-dihydroxycinnamonitrile derivatives acting as dual-reactive probes readily convertible to highly fluorescent 7-hydroxy-2-iminocoumarin scaffolds upon reacting with the two selected enzymes, were synthesised. The two phenolic groups available within the core structure of these probes play a pivotal role in generating iminocoumarin scaffold through an intramolecular cyclisation reaction (hydroxyl group in C-2 position) and in enhancing its push-pull character (hydroxyl group in C-4 position). Their orthogonal and temporary protection with two different enzyme-labile masking groups is the cornerstone in the design of this novel class of fluorogenic "turn-on" probes. Their evaluation using fluorescence-based *in vitro* assays and HPLC-fluorescence/-MS analyses have enabled us both to demonstrate the claimed activation mechanism (in particular the specific order in which the two enzymes react with the probe) and to highlight the potential utility of these advanced chemical tools in multi-analyte sensing applications.

Introduction

Fluorescent probes for dual- or multi-analyte detection have recently emerged as valuable and powerful analytical tools for (bio)sensing and bioimaging applications.[1] Indeed, they have clear advantages over single-analyte responsive probes used either sequentially or simultaneously, particularly to improve the power of analytic assays (in terms of throughput, spatial/temporal resolution and quantitative response) but also facilitate their implementation in molecular-scale devices, through atom economy and high-density integration.[2] Among the host of useful and promising applications recently claimed for such fluorescent molecular logic gates, "smart" medical diagnostics relying on the simultaneous quantitative detection of several analytes to one pathology is often highlighted.[3] The design principle commonly adopted for constructing such fluorescent chemodosimeters/chemosensors is based on the incorporation of multiple reacting/binding sites within a unique photoactive molecular scaffold. That is able to generate different fluorescence output signals (distinct spectral responses *via* the same or different wavelength channels) upon its reaction with several distinct analytes. Molecular probes with a single reacting/binding site, capable of producing distinct optical outputs depending on the analyte being reacted, are also considered. Thus, numerous dual fluorogenic probes for sensing two species from among some metal cations, anions and/or neutral biorelevant molecules (*e.g.*, biothiols), through specific chemical reactions occurring either simultaneously or consecutively, have been reported during the last decade.[1] By contrast, little attention has been paid to reaction-based fluorescent probes enabling two different enzyme activities monitoring within the same sample or biological medium, whereas they are valuable tools to improve the diagnosis accuracy (avoiding false positives) and expedite the right treatment of diseases. Particularly noteworthy are two examples of FRET-based probe for simultaneous (or sequential) intracellular detection of β-D-glucosidase and phosphodiesterase I, and two distinct proteases namely matrix metalloproteinase 2 (MMP-2) and caspase-3, respectively.[4] This probe principle may find further applications in multi-enzyme analysis by changing the sensitive linkers and/or fluorophore pairs, but will remain limited to hydrolases detection. More recently, a brilliant concept for *in vivo* bioluminescence detection of H_2O_2 and caspase-8 (an

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Electronic Supplementary Information (ESI) available: [Synthetic procedures for Ac-PHBA and PhAc-PABA, experimental details of *in vitro* enzyme assays and all analytical data]. See DOI: 10.1039/x0xx00000x

apoptosis-related cysteine protease), two major contributors to oxidative stress and cell death in injury and disease, has been reported by Bertozzi, Chang and co-workers.[5] *In situ* formation of D-luciferin from two caged precursors, each specifically activated by one of the two targeted analytes, and through the CBT-based "click" reaction[6], is the cornerstone of this dual-analyte imaging strategy. Interestingly, the recent availability of enzyme-responsive caged luciferins (*i.e.*, luciferin derivatives whose the 6-NH_2 or 6-OH is masked by an enzyme-labile moiety)[7,8, 9, 10] should facilitate the further extension of this "self-assembly" bioluminogenic approach to simultaneous imaging of two relevant enzymes in living systems.

In this context, we are interested in devising a new fluorogenic platform engineered to respond only in the presence of two distinct enzymes and to generate an intense "turn-on" fluorescence signal as a result of domino reactions triggered by these two analytes. Indeed, it is now well established that reaction-based small-molecule fluorescent probes (also known as latent fluorophores, pro-fluorophores or fluorescent chemodosimeters) are powerful tools for detecting/imaging a myriad of analytes including enzymes, biomolecules, chemical pollutants, cations and anions.[11, 12] Among the various organic reaction-based strategies currently implemented in the field of molecular (bio)sensing, the "covalent assembly" approach[13] using the analyte of interest to carry out the fluorophore synthesis *in situ* from non-fluorescent caged precursor(s), is a very promising way to readily obtain highly fluorimetric "turn-on" signal from zero background. Thus, this concept was successfully applied for the detection of various analytes including enzymes (monoamine oxidases (MAO A and B), penicillin G acylase (PGA), phosphatases), cations/anions (Cu(II), Hg(II), MeHg(I), F^- and NO_2^-), sarin mimics, H_2S, NO and peroxynitrite, through the formation of pyrrolo-coumarin, pyrazino-benz[*e*]indole, 7-*N*,*N*-dialkylaminocoumarin, 2-arylbenzotriazole, pyronin B, benzo[*c*]cinnoline and resorufin fluorescent scaffolds.[13, 14,15] Further implementation of this strategy to fluorogenic bimolecular reactions producing red-emitting cyanine dyes, was recently reported for illuminating G-quadruplex DNA structures[16] and protein CRABPII.[17] It is also worth mentioning that the more traditional strategy based on protection-deprotection of the key functional group of a fluorophore (typically, a phenol or an aniline moiety)[12] was recently used by Prost and Hasserodt to develop the appealing "double gating" concept.[18] The activation by successive cleavage of a pre-pro-fluorophore with two distinct enzymes namely β-D-galactosidase and leucine aminopeptidase leads to the release of a phenol-based fluorophore.

From our side, we wished to apply the "covalent assembly" probe design principle to reach the same purpose. To this end, a fluorophore precursor bearing two distinct reactive sites, one involved in the formation of the conjugated backbone and one for enhancing the push-pull character, masked by two different enzyme-labile protecting groups, is needed (Figure 1). The most convenient class of fluorophores to be used to rapidly provide a proof-of-concept is undoubtedly the 7-amino/7-hydroxy-(2-imino)coumarins family.[19] Indeed, non-fluorescent precursors readily convertible into (2-imino)coumarins through a specific cyclisation reaction triggered by a reactive analyte are already well known.[14b, 14c, 14e-g, 14i-k, 14m, 14o-r] Furthermore, since 7-amino-/7-hydroxy-(2-imino)coumarins are known to be fluorogenic dyes (7-NH_2/7-OH moiety acting as a fluorescence "OFF-ON" switch based on its protection-deprotection), one would assume that further functionalization of the (2-imino)coumarin precursor with an aniline or a phenol moiety masked by a trigger recognition unit reactive toward a second distinct analyte could easily lead to reaction-based probes acting as "AND" fluorogenic logic gates (Figure 1).[3,20]

In this paper, we report the practical implementation of this innovative dual-sensing strategy using hydrolase (PGA or pig liver esterase (PLE)) and nitroreductase (NTR) as enzyme pair model. This had led to the preparation of three dual-reactive probes whose the activation mechanism was studied in detail through *in vitro* fluorescence assays and HPLC-fluorescence/-MS analyses, particularly to demonstrate *in situ* formation of blue-emitting 2-iminocoumarin scaffolds mediated by combination of the two enzymes.

Results and discussion

General considerations for the design of dual-enzyme fluorogenic "turn-on" probes - Selection of structural units

As suggested above, we have decided to focus on the design of molecular probes capable of generating a fluorescent dye belonging to the family of 3-substituted-7-hydroxy-2-iminocoumarins, upon dual-enzymatic activation. Indeed, 7-hydroxycoumarin derivatives are often more fluorescent than their 7-amino counterparts, particularly at physiological pH, and the facile functionalization of their C-3 position with an electron-withdrawing group (typically, a cyano group) or an heteroaryl substituent (*e.g.*, 2-benzothiazolyl or 2-benzimidazolyl moiety) is an asset to red-shift their absorption/emission maxima, and to facilitate fluorescence detection in complex biological media.[21, 22] Furthermore, bis-*O*-protected 2,4-dihydroxycinnamonitrile derivatives should be easily synthesised from cheap commercial starting materials (*i.e.*, 2,4-dihydroxybenzaldehyde and malonitrile or benzothiazole-2-acetonitrile) and through a Knoevenagel-type condensation reaction. Consequently, the two fluorophores namely 3-(2-benzothiazolyl)-7-hydroxy-2-iminocoumarin[23] and 3-cyano-7-hydroxy-2-iminocoumarin[24, 25] have been selected as fluorescent reporters for this study (Figure 1). For *in vitro* validations performed under simulated physiological conditions (*i.e.*, PB, pH 7.5), the significant hydrophobic character of the claimed coumarin precursor is a potential issue. So, we had initially planned to design fluorogenic "turn-on" probes reactive toward both NTR and a sulfatase. Indeed, this latter class of hydrolytic enzymes is known to catalyse the hydrolysis of sulfate esters whose the inherent hydrophilicity is beneficial for the overall aqueous solubility of the probes.[26] Unfortunately, our preliminary works have revealed that dual NTR-sulfatase fluorogenic "turn-on" probes are completely

unstable in aqueous solution and a rapid hydrolysis of their aryl sulfate ester moiety occurred. Alternatively, we have chosen to work with (i) PGA (also known as penicillin amidase) a commercially available and widely used biocatalyst in the enzymatic synthesis of β-lactam antibiotics, since it allows for the deprotection of phenylacetyl-protected amines[27], and (ii) pig liver esterase (PLE), known to catalyse the hydrolysis of acetate esters and commonly used for kinetics resolution and asymmetric synthesis in organic chemistry[28], in place of sulfatases. However, the use of these two model enzymes directly impacts the structural requirements of reaction-based probes. Indeed, to transfer the amide bond-breaking event to a phenol release, the use of a self-immolative spacer is required.[29] *para*-Aminobenzyl alcohol (PABA) was chosen because it has been successfully used to conjugate peptide substrates to masked fluorophores, to obtain valuable fluorogenic reagents for protease assays and/or *in vivo* molecular imaging applications.[30] Thus, *para*-(phenylacetamido)benzyl moiety was selected as the PGA-labile phenol protecting group. To enhance the overall stability of the phenol-based probes bearing an esterase-sensitive moiety, it is also recommended to introduce a self-immolative spacer (typically, *para*-hydroxybenzyl alcohol (PHBA)) between the acetyl and phenol moieties. Consequently, *para*-(acetoxy)benzyl (Ac-PHBA) was selected as the PLE-labile phenol protecting group.[8, 31] In both cases, the bio-labile moiety will be introduced onto the phenol *via* a carbonate linkage because preliminary etherification-based synthetic strategies failed to give the desired protected phenol derivatives (*vide infra*). Finally, we have regarded the masking of the second phenol function by the *para*-nitrobenzyl moiety, which is a commonly used caging group that targets reductase activity for sensor and prodrug development.[10, 32] At this stage, it is also important to point out the preferred phenolic position for introducing each enzyme-labile trigger-spacer combined unit: 2-OH for *para*-nitrobenzyl and 4-OH for *para*-(phenylacetamido)benzyl and *para*-(acetoxy)benzyl, arising from our preliminary synthetic attempts and shortcomings encountered (Figure 1).

Synthesis of bis-*O*-protected 2,4-dihydroxycinnamonitrile derivatives

The three dual-enzyme fluorogenic "turn-on" probes were synthesised in four steps (Scheme 1). First, the 4-OH group of 2,4-dihydroxybenzaldehyde was selectively protected as silyl ether using standard conditions. The resulting TBDMS ether **1** was next reacted with an excess of *para*-nitrobenzyl bromide (5 equiv.) under mild "Kuhn methylation" conditions (*i.e.*, the use of Ag_2O as electrophilic catalyst and base)[33], to readily introduce the NTR-labile protecting group into the 2-OH position, without premature deprotection of the 4-OH group. The full-protected benzaldehyde derivative **2** was condensed with either benzothiazole-2-acetonitrile or malonitrile in EtOH and in the presence of a catalytic amount of piperidine, to give the Knoevenagel adducts which were desilylated by treatment with TBAF in THF. The corresponding phenols **3** and **4** were obtained in satisfying yields (38% and 55% for the two steps, respectively). Pre-activation of Ac-PHBA was achieved by treatment with diphosgene in dry DCM in presence of TEA. The resulting crude chloroformate was reacted with **3** to provide the targeted dual PLE-NTR fluorogenic "turn-on" probe **5** in 32% yield. Interestingly, this benzothiazolyl-based probe **5** was obtained as a mixture of E/Z isomers (56 : 44 and 85 : 15 determined by HPLC and NMR respectively ; difference in this ratio value was explained by the lower solubility of less polar isomer in RP-HPLC eluents and different UV-vis absorption properties between two isomers (both wavelength maximum and molar extinction coefficient)). This synthetic methodology was also applied to PhAc-PABA but failed (due to the premature degradation of PhAc-PABA throughout its activation with diphosgene). No further attempts were made to obtain PGA-NTR benzothiazolyl-based probe because we suspected that its high hydrophobic character would be prohibitive for applications in aqueous buffers. For the dicyano probe **8** and **9**, diphosgene was first reacted with phenol **4** and benzyl alcohol Ac-PHBA/PhAc-PABA was next introduced (particularly to avoid degradation of PhAc-PABA) but the desired products were recovered in poor yields (11% and 9% respectively). These results were unsatisfactory, so pre-activation of Ac-PHBA/PhAc-PABA with *para*-nitrophenyl chloroformate in dry THF in the presence of TEA was investigated. The resulting *para*-nitrophenyl carbonate derivatives **6**[34] and **7**[35] were purified and reacted with **4** to provide the two targeted dual fluorogenic "turn-on" probes **8** and **9** with improved 30% and 17% yields respectively. All spectroscopic data (see Experimental section and ESI†), especially IR, NMR and mass spectrometry, were in agreement with the structures assigned. Their purity was checked by RP-HPLC and found to be in the range 94-96%. It was not feasible to perform further RP-HPLC purification (typically, the method best suited to increase this percentage purity to 100%) due to hydrophobicity and moderate stability of **5**, **8** and **9** in aqueous acidic mobile phases.

Photophysical characterization and dual-enzymatic activation of fluorogenic "turn-on" probes - Fluorescence-based assays

UV-vis absorption spectra of compounds **5**, **8** and **9** were recorded in phosphate buffer (PB, 0.1 M, pH 7.5, simulated physiological conditions).[36] They exhibited a broad absorption spectrum covering virtually the entire UV spectral range (250-450 nm), and characterized by a maximum centered at 372-383 nm depending on the nature of C-3 substituent (see Figure S1†). This spectral pattern is in part due to the formation of aggregates promoted by the hydrophobic character of these cinnamonitrile derivatives. This aggregation behaviour prevented us from obtaining linear relationships between absorbance and concentration required to determine molar extinction coefficients of these absorbing species. As expected, these three probes, without exception, were not fluorescent under simulated physiological conditions and their emission spectrum exhibits a zero-baseline remaining constant over time (see Figures S4-S6†).

Concerning the dual-enzymatic activation of **5**, **8** and **9**, fluorescence-based *in vitro* assays were performed with commercial enzymes (recombinant NTR from *E. coli* used with co-factor NADH, recombinant PGA from *Alcaligenes faecalis* and esterase from porcine liver). We were mindful that fluorescence of NADH (Abs./Em. maxima = 340/470 nm)[37] may cause interference with enzyme-induced fluorogenic signal but the excitation channel selected for the probes (455 nm for **5** and 418 nm for **8** and **9**) is far enough away from absorption maximum of co-factor to prevent its photonic excitation and subsequent emission. For each pro-fluorophore, different scenarios were tested under simulated physiological conditions (PB, pH 7.5, 37 °C). Concerning benzothiazolyl-based probe **5**, fluorescence emission time-course measurements were achieved with the following enzyme addition procedures: (i) NTR/NADH (20 min) then PLE, (ii) PLE (10 min) then NTR/NADH, (iii) NTR/NADH and PLE simultaneously, and without enzyme (Figure 2A). We also checked that co-factor alone did not trigger phenol deprotection resulting in fluorogenic response, by carrying out some assays that involved pre-incubation of probe with NADH before adding enzymes. A rapid and gradual increase of blue-green fluorescence intensity at 489 nm (Ex. 455 nm) fully consistent with *in situ* formation of 3-(2-benzothiazolyl)-7-hydroxy-2-iminocoumarin was observed when **5** is pre-incubated with PLE before adding NADH and NTR. It was somewhat disappointing to note that probe's activation was much less effective with two other incubation protocols. These results suggested that this full-protected 2,4-dihydroxycinnamonitrile derivative may be a poor substrate of NTR or act as an effective competitive inhibitor of this enzyme, and is reactive toward this reductase only once its 4-OH phenol group is free. Curiously, a slight increase in fluorescence was observed when the probe is pre-incubated only with NTR/NADH. The presence of a minute amount of free 4-OH 2,4-dihydroxycinnamonitrile derivative **3** in the sample (reminder: purity 96%) or its minor formation in PB at pH 7.5 was suspected to explain this premature fluorescent activation by a single enzyme. However, the lack of fluorescence increase observed for the control reaction involving incubation of **5** with buffer alone, illustrates one of the major benefits of the dual-reactive fluorogenic probes compared to pro-fluorophores activated through a single (bio)chemical event. Even though the overall stability of such fluorogenic platforms may not necessarily be remarkable under physiological conditions, the probability of obtaining false positive fluorescence signals is lower because this would require the non-specific hydrolysis of their two enzyme-responsive caging/quenching groups. Better results were obtained with cyano-based probes **8** and **9**. Time-dependant fluorescence analyses were performed under the same conditions than those described above, except for the excitation/emission wavelength channel (Ex./Em. 418/458 nm), which was tuned for an optimal detection of 3-cyano-7-hydroxy-2-iminocoumarin (Figure 2B-C). Furthermore, for the 4-*O*-(*para*-(phenylacetamido)benzyloxycarbonyl) derivative **9**, PGA was used instead of PLE. In both cases, a strong blue fluorescence activation monitoring the formation of 7-hydroxy-2-iminocoumarin scaffold was observed when **8** and **9** were incubated with both enzymes simultaneously or better according the sequential mode: pre-incubation with hydrolase (10 min) followed by addition of NTR/NADH. Only the sequential assay involving pre-incubation of the probes with NTR led to poor fluorescence activation. Interestingly, further addition of compound **4** (a good fluorogenic substrate of NTR) into this latter enzymatic reaction mixture did not led to a dramatic change in fluorescence signal (see Figure S7†). These results supports the hypothesis that compounds **5** and **8** are effective at blocking the activity of this reductase. However, a deeper understanding of this inhibition mechanism requires further enzyme kinetics and molecular docking experiments that will soon be carried out and reported in due course.

To confirm that the resulting fluorescence emission was due to 7-hydroxy-2-iminocoumarin dye formed *in situ*, all enzymatic reaction mixtures were directly analysed by RP-HPLC coupled with fluorescence detection using aqueous triethylammonium bicarbonate buffer (TEAB, 50 mM, pH 7.5) and CH_3CN as eluents (see Figures 3 and S9† for illustrative examples of RP-HPLC elution profiles and Figure S8† for negative controls with starting non-fluorescent probes). The use of a neutral aqueous mobile phase is required to ensure phenol deprotonation of newly formed 7-hydroxy-2-iminocoumarins and maximize their fluorescence properties and thus their detection. Furthermore, the facile synthesis of authentic samples of 3-(2-benzothiazolyl)-7-hydroxy-2-iminocoumarin[23], 3-(2-benzothiazolyl)-7-hydroxycoumarin[23], 3-cyano-7-hydroxy-2-iminocoumarin and 3-cyano-7-hydroxycoumarin[24, 25], has enabled us to have reference compounds for such liquid chromatographic analyses. Thus, the identification of peaks was done by comparison of retention times with those of standards. In the case of benzothiazolyl-based probe **5**, the formation of both 3-(2-benzothiazolyl)-7-hydroxy-2-iminocoumarin and 3-(2-benzothiazolyl)-7-hydroxycoumarin was confirmed (Table 1, entries 1 and 2). The side-formation of coumarin through the non-enzymatic hydrolysis of the imine moiety occurred during the enzymatic reaction and HPLC analysis. This is not a problem for the claimed dual-enzyme sensing scheme because this also a strongly emissive species (Table 1, entries 1 and 2). For bis-cyano-based probes **8** and **9**, unambiguous identification of 3-cyano-7-hydroxy-(2-imino)coumarins was also done but a further minor broad split peak was observed on the RP-HPLC elution profiles, which may assigned to 3-carbamoyl-7-hydroxy-(2-imino)coumarins (Figure S9† and Table 1, entries 3-5). Indeed, the nitrile moiety introduced onto the C-3 position of coumarins, is prone to facile hydrolysis.[24] The non-enzymatic conversion of 2-iminocoumarin derivative into the corresponding coumarin is particularly beneficial because the relative fluorescence quantum yield of this latter product was found to be significantly higher than that of its parent imine (Table 1, entries 3-5).

Dual-enzymatic activation of fluorogenic "turn-on" probes - Mechanism study through RP-HPLC-MS analyses

To confirm the sensing process claimed in Figure 1, HPLC-MS analyses of the enzymatic hydrolysis mixtures were performed. To carry out mass detection through the "full-scan" detection mode (more practical and convenient but less sensitive than the single-ion monitoring (SIM) mode), enzyme reactions were performed at a higher concentration than this previously used for fluorescence-based assays (85 μM against 1.0 μM). It was not possible to get reproducible results with the benzothiazolyl-based probe **5** due to its poor solubility in PB at this concentration. Consequently, such mechanistic study was focused only on less hydrophobic bis-cyano-based probes **8** and **9** (Figures 4 and S10-S12†). The first valuable information disclosed by these HPLC-MS analyses concerned the stability of the probes in the reaction buffer. Indeed, as shown in Figures 4B and S10†, following incubation at pH 7.5 without enzymes, probes **8** and **9** were partly degraded to a single product identified as the corresponding benzaldehyde derivative (Table 1, entries 6 and 7). Under these mild conditions, retro-Knoevenagel reaction is able to occur but curiously, such side-reaction was never reported in publications describing *O*-protected 2-hydroxycinnamonitrile derivatives as effective reaction-based fluorescent probes.[38] This does not challenge detection mechanisms based on *in situ* formation of 2-iminocoumarins because the formed benzaldehyde-type derivative remains non-fluorescent, but it is important to bear in mind that this may prevent quantitative determination of targeted analyte through the decrease of the "active" probe concentration. In the future, this should be addressed in studies focused on the use of benzylidenemalonitrile-based probes as chemodosimeters. Gratifyingly, no trace of free 2-OH and/or 4-OH 2,4-dihydroxycinnamonitrile derivatives that might lead to 7-hydroxy-(2-imino)coumarins without enzymatic action were detected in these blank samples. This was a bit in contrast with conclusions drawn from fluorescent measurements related to enzyme assays involving pre-incubation with NTR/NADH followed by addition of hydrolase (*vide supra*), but explained by the differences in probe's concentration used in both assays (85 μM against 1.0 μM). The preferential reaction of probes with hydrolase against NTR was clearly demonstrated through the detection of 2,4-dihydroxycinnamonitrile derivative having free 4-OH and 2-OH always masked as *para*-nitrobenzyl ether (Figure 4C and Table 1, entry 8). A similar trend was observed with the retro-Knoevenagel products as evidenced by the detection of 2-*O*-(*para*-nitrobenzyl)-2,4-dihydroxybenzladehyde (Figure 4D and Table 1, entry 9). The intermediate was next deprotected onto their 2-OH position under the action of NTR/NADH, to undergo rapid spontaneous cyclisation leading to 7-hydroxy-(2-imino)coumarins bearing either the cyano or carbamoyl group as C-3 substituent (Figure 4D and table 1, entries 3-5). We were therefore able to demonstrate that *in situ* synthesis of the fluorophore scaffold is really triggered by two distinct enzyme activities through a sequential mode of action.

Conclusions

For the first time, we have demonstrated that the "covalent-assembly" probe design principle could be successfully applied for the simultaneous fluorogenic detection of two different enzyme activities namely hydrolase and reductase. The double activation of these reactive probes through hydrolysis of amide (or ester) bond and nitro-reduction, followed by domino 1,6-elimination/cyclisation reactions, was efficient at generating strongly fluorescent 7-hydroxy-(2-imino)coumarins. These sophisticated biosensing systems act as AND-type molecular logic gates because fluorescence signal production occurred only in the presence of both analytes. To rapidly apply this promising concept to molecular imaging of disease-biomarkers pairs for diagnostic purposes, extension of the "doubly-triggered covalent assembly" approach to longer wavelength fluorophores (*e. g.*, pyronin B or far-red cyanine dyes[39]) is currently in progress in our lab. Furthermore, the dual-reactive 7-hydroxy-(2-imino)coumarin precursors of this work can be easily modified to rapidly obtain "dual-lock" structures combining two distinct reactive/quenching groups sensitive to the same analyte. Indeed, this is the preferred approach for designing reaction-based fluorescent probes with unprecedented performances, especially those related to the effective discrimination between molecules exhibiting similar chemical reactivity (*e.g.*, biothiols).[40,41]

Experimental section

General

Unless otherwise noted, all commercially available reagents and solvents were used without further purification. TLC were carried out on Merck DC Kieselgel 60 F-254 aluminum sheets. The spots were directly visualised or through illumination with UV lamp (λ = 254/365 nm) and/or staining with a phosphomolybdic acid solution (4.8% wt. in EtOH). Column chromatography purifications were performed on silica gel (63-200 μm) from Sigma-Aldrich (technical grade). All solvents were dried by standard procedures [DCM: distillation from CaH_2; absolute EtOH: storage over anhydrous Na_2SO_4; THF: distillation from sodium benzophenone diketyl; and TEA: storage over anhydrous Na_2SO_4]. Anhydrous DMF was purchased from Carlo Erba, and stored over 4 Å molecular sieves. Peptide synthesis grade DIEA was provided by Iris Biotech GmbH. The HPLC-gradient grade acetonitrile (CH_3CN) was obtained from Carlo Erba. Formic acid (FA, grade "eluent additive for LC-MS") was provided by Sigma-Aldrich. Phosphate buffer (100 mM, pH 7.5) and aq. mobile-phases for HPLC were prepared using water purified with a PURELAB Ultra system from ELGA (purified to 18.2 MΩ.cm). PLE (esterase from porcine liver, E3019, 27 U/mg of lyophilized enzyme), NTR (from *E. coli*, N9284, 0.1 U/μg, lyophilized enzyme + buffer resuspended in ultrapure water) and NADH were purchased from Sigma-Aldrich and stored at -20 °C. Recombinant PGA (from *Alcaligenes faecalis*) was provided by Universität Hohenheim (0.63 U/mg of lyophilized enzyme). Triethylammonium bicarbonate (TEAB, 1.0 M) buffer was prepared from distilled TEA and CO_2 gas. 3-(2-Benzothiazolyl)-

7-hydroxycoumarin, 3-(2-benzothiazolyl)-7-hydroxy-2-iminocoumarin, 3-cyano-7-hydroxycoumarin, 3-cyano-7-hydroxy-2-iminocoumarin were prepared according to literature procedures.[23-25] See ESI† for the synthesis of Ac-PHBA and PhAc-PABA.

Instrument and methods

Centrifugation steps were performed with an Hettich Universal 320 instrument. ^{1}H- and ^{13}C-NMR spectra of compounds **1-9** were recorded either on a Bruker Avance 300 or on a Bruker Avance 500 spectrometer. Chemical shifts are expressed in parts per million (ppm) from the residual non-deuterated solvent signal.[42] *J* values are expressed in Hz. IR spectra were recorded with a Bruker Alpha FT-IR spectrometer equipped with an universal ATR sampling accessory. The bond vibration frequencies are expressed in reciprocal centimeters (cm^{-1}). HPLC-MS analyses were performed on a Thermo-Dionex Ultimate 3000 instrument (pump + autosampler) equipped with a diode array detector (Thermo-Dionex DAD 3000-RS) and a MSQ Plus single quadrupole mass spectrometer (LRMS analyses through ESI). HPLC-fluorescence analyses were performed with the same instrument coupled to a RS fluorescence detector (Thermo-Dionex, FLD 3400-RS). High-resolution mass spectra (HRMS) were recorded on a Thermo LTQ Orbitrap XL apparatus equipped with an electrospray ionisation (ESI) source. UV-visible spectra were obtained either on a Varian Cary 50 scan spectrophotometer by using a rectangular quartz cell (Hellma, 100-QS, 45 × 12.5 × 12.5 mm, pathlength 10 mm, chamber volume: 3.5 mL). Fluorescence spectroscopic studies (emission/excitation spectra and kinetics) were performed with an HORIBA Jobin Yvon Fluorolog spectrophotometer (software FluoEssence) with a standard fluorometer cell (Labbox, LB Q, 10 mm). Emission spectra were recorded under the same conditions after excitation at the corresponding wavelength (shutter: Auto Open, excitation slit = 5 nm and emission slit = 5 nm). All fluorescence spectra were corrected.

Fluorescence quantum yields of 3-cyano-7-hydroxycoumarin and 3-cyano-7-hydroxy-2-iminocoumarin were measured at 25 °C by a relative method using quinine sulfate (Φ_F = 60% in 0.05 M H_2SO_4) as a standard (for the corresponding Abs/Ex/Em spectra, see Figures S2-S3†).[43] The following equation was used to determine the relative fluorescence quantum yield:

$$\Phi_F(x) = (A_S/A_X)(F_X/F_S)(n_X/n_S)^2\Phi_F(s)$$

where A is the absorbance (in the range of 0.01-0.1 A.U.), F is the area under the emission curve, n is the refractive index of the solvents (at 25 °C) used in measurements, and the subscripts s and x represent standard and unknown, respectively. The following refractive index value is used: 1.333 for aq. H_2SO_4 and 1.337 for PB.

High-performance liquid chromatography separations

Several chromatographic systems were used for the analytical experiments (HPLC-MS or HPLC-fluorescence): System A: RP-HPLC-MS (Phenomenex Kinetex C_{18} column, 2.6 µm, 2.1 × 50 mm) with CH_3CN (+ 0.1% FA) and 0.1% aq. FA (pH 3.2) as eluents [linear gradient from 5% to 100% (5 min) of CH_3CN followed by isochratic at 100% (1.5 min)] at a flow rate of 0.5 mL min^{-1}. UV-visible detection was achieved at 220, 260, 300 and 360 nm (+ diode array detection in the range 220-500 nm). ESI-MS detection in the positive/negative mode ("full scan", 150-1500 a.m.u., data type: centroid, needle voltage: 3.0 kV, detector voltage: 1100 V, probe temperature: 350 °C, cone voltage: 75 V and scan time: 1 s). System B: System A with 100-700 a.m.u for "full scan" mass detection. System C: System A with the following gradient [0% CH_3CN (2 min) followed by linear gradient from 0% to 100% (6 min) of CH_3CN followed by isochratic at 100% (1 min)]. UV-visible detection was achieved at 220, 260, 350 and 418 nm (+ diode array detection in the range 220-500 nm). System D: RP-HPLC-fluorescence (Phenomenex Kinetex C_{18} column, 2.6 µm, 2.1 × 50 mm) with CH_3CN and aq. TEAB (50 mM, pH 7.5) as eluents [0% CH_3CN (1 min) followed by linear gradient from 0% to 100% (5 min) of CH_3CN followed by isochratic at 100%] at a flow rate of 0.5 mL min^{-1}. Fluorescence detection was achieved at 45 °C at the following Ex. /Em. channels: 350/460 nm and 418/458 nm (sensitivity: 1, PMT 1, filter wheel: auto). System E: System D with the following Ex./Em. channels for fluorescence detection: 350/460 nm, 431/488 nm and 455/489 nm.

4-((*tert*-Butyldimethylsilyl)oxy)-2-hydroxybenzaldehyde (1).[44] Under Ar atmosphere, to a stirred solution of TBDMS-Cl (2.20 g, 14.4 mmol, 1 equiv.) in dry DCM (40 mL) was added imidazole (1.15 g, 15.8 mmol, 1.1 equiv.). After 5 min of stirring, 2,4-dihydroxybenzaldehyde (2 g, 14.4 mmol, 1 equiv.) was added and the resulting reaction mixture was stirred at RT for 4 h. Thereafter, the reaction mixture was washed with deionised water and brine, dried over anhydrous $MgSO_4$, and concentrated under vacuum. The resulting residue was purified by chromatography on a silica gel column (PE-EtOAc, step gradient from 100 to 95 : 5, v/v) to give the desired silyl ether **1** as colourless oil (2.62 g, yield 72%). R_f 0.61 (PE-EtOAc, 98 : 2, v/v); δ_H(300 MHz, $CDCl_3$) 11.33 (s, 1 H), 9.72 (s, 1 H), 7.40 (d, *J* 8.7, 1 H), 6.46 (dd, *J* 8.7, *J* 2.4, 1 H), 6.38 (d, *J* 2.4, 1 H), 0.98 (s, 9 H), 0.25 (s, 6 H).

4-((*tert*-Butyldimethylsilyl)oxy)-2-((4-nitrobenzyl)oxy)-benzaldehyde (2). To a stirred solution of phenol **1** (1 g, 3.96 mmol, 1 equiv.) in Et_2O (17 mL) were sequentially added 4-nitrobenzyl bromide (4.28 g, 19.8 mmol, 5 equiv.) and Ag_2O (3.6 g, 15.8 mmol, 4 equiv.) at RT. The resulting reaction mixture was stirred overnight. Thereafter, the crude was directly purified by chromatography on a silica gel column (eluent: PE-EtOAc, step gradient from 100 to 90.7 : 0.3, v/v to completely remove the large excess of 4-nitrobenzyl bromide and after a step gradient from 95 : 5 to 85 : 15, v/v) to afford the desired product **2** as white solid (1.35 g, yield 88%). R_f 0.78 (DCM); ν_{max}(neat)/cm^{-1} 2952, 2927, 2855, 1683, 1593, 1340, 1256; δ_H(300 MHz, $CDCl_3$) 10.38 (s, 1 H), 8.28 (d, *J* 9.0, 2 H),

7.79 (d, *J* 8.7, 1 H), 7.63 (d, *J* 8.7, 2 H), 6.53 (dd, *J* 9.3, *J* 2.1, 1 H), 6.39 (d, *J* 2.4, 1 H), 5.24 (s, 2 H), 0.96 (s, 9 H), 0.21 (s, 6 H); δ_C(75 MHz, $CDCl_3$) 187.9, 163.0, 161.8, 147.7, 143.4, 131.0, 127.5, 124.0, 119.6, 113.5, 104.5, 69.0, 25.5, 18.3, -4.3; HPLC (system A): t_R = 6.2 min, purity = 100% (at 260 nm); λ_{max} (recorded during the HPLC analysis)/nm 227, 272 and 306; LRMS (ESI+): *m/z* 388.3 $[M + H]^+$ (100) and 641.1 $[M + H + CH_3CN]^+$ (80), calcd for $C_{20}H_{25}NO_5Si$ 387.1.

2-(Benzothiazol-2-yl)-3-(4-hydroxy-2-((4-nitrobenzyl)oxy)-phenyl)acrylonitrile (3). Benzaldehyde **2** (360 mg, 0.93 mmol, 1 equiv.) and benzothiazole-2-acetonitrile (162 mg, 0.93 mmol, 1 equiv.) were dissolved in absolute EtOH (46 mL). After, 1 drop of piperidine was added and the reaction mixture was stirred at RT for 2 h. Thereafter, volatiles were removed under vacuum and the resulting residue was dissolved in THF (10 mL). The mixture was cooled to 0 °C with an ice-water bath. Then TBAF (1.0 M in THF, 1 mL) was added and the mixture was left to warm at RT for 40 min. Thereafter, the reaction mixture was quenched by adding glacial acetic acid (0.5 mL) and solvents were evaporated under reduced pressure. The resulting residue was purified by chromatography on a silica gel column (eluent: EtOAc-MeOH-TEA, step gradient from 8 : 2 : 0 to 8 : 1.9 : 0.1, v/v). The product was taken with EtOAC, then acidified with two drops of glacial acid acetic and washed with deionised water in order to remove TEA, the organic layer dried over anhydrous Na_2SO_4, filtered and concentrated to obtain the desired product 3 as orange solid (150 mg, yield 38%, mixture of E and Z isomers). R_f 0.27 (DCM-EtOAc, 9.5 : 0.5, v/v); ν_{max}(neat)/cm^{-1} 3358, 2925, 2216, 1620, 1579, 1522, 1367,1345, 1299; δ_H(300 MHz, DMSO-d_6) 10.70 (bs, 1 H, OH), 8.56 (s, 1 H), 8.32 (d, *J* 7.8, 2 H), 8.21 (d, *J* 8.1, 1 H), 8.13 (d, *J* 7.2, 1 H), 8.02 (d, *J* 7.5, 1 H), 7.78 (d, *J* 7.8, 2 H), 7.56-7.47 (m, 2 H), 6.64 (m, 2 H), 5.44 (s, 2 H); δ_C(75 MHz, DMSO-d_6) 177.4, 164.5, 160.0, 153.6, 147.7, 144.8, 141.7, 134.3, 130.3, 128.6, 127.5, 126.3, 124.3, 123.3, 122.9, 117.5, 113.1, 110.0, 101.3, 100.6, 69.4; HPLC (system B): t_R = 5.8 min, purity = 100% (at 260 nm); λ_{max} (recorded during the HPLC analysis)/nm 221, 262 and 395; LRMS (ESI-): *m/z* 428.1 $[M - H]^-$ (100), calcd for $C_{23}H_{15}N_3O_4S$ 429.1.

2-(4-Hydroxy-2-((4-nitrobenzyl)oxy)benzylidene)-malononitrile (4). Benzaldehyde **2** (1 g, 2.58 mmol, 1 equiv.) and malonitrile (180 mg, 2.84 mmol, 1.05 equiv.) were dissolved in absolute EtOH (130 mL). After, 1 drop of piperidine was added and the reaction mixture was stirred at RT for 1 h. thereafter, volatiles were removed under vacuum and the resulting residue was dissolved in THF (26 mL). The mixture was cooled to 0 °C with an ice-water bath. Then TBAF (1.0 M in THF, 2.85 mL) was added and the mixture was left to warm at RT for 50 min. Thereafter, the reaction mixture was quenched by adding glacial acetic acid (0.4 mL) and solvents were evaporated under reduced pressure. The resulting residue was dissolved in DCM and washed with aq. 1.0 M HCl (20 mL) and brine (20 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum. The resulting residue was purified by trituration in MeOH and filtration to obtain the desired product **4** as orange solid (460 mg, yield 55%). R_f 0.5 (DCM-EtOAc, 9 : 1, v/v); ν_{max}(neat)/cm^{-1} 3335, 2221, 1605, 1561, 1513, 1354, 1329, 1289; δ_H(300 MHz, DMSO-d_6) 11.10 (bs, 1 H, OH), 8.31 (s, 1 H), 8.28 (d, *J* 8.7, 2 H), 8.05 (d, *J* 9.0, 1 H), 7.77 (d, *J* 8.7, 2 H), 6.62 (dd, *J* 8.7, *J* 2.1, 1 H), 6.57 (d, *J* 2.1, 1 H), 5.40 (s, 2 H); δ_C(125 MHz, DMSO-d_6) 166.6, 160.6, 153.9, 147.7, 144.27, 131.0, 120.9, 124.1, 116.0, 115.0, 112.6, 110.4, 101.0, 74.9, 69.4; HPLC (system B): t_R = 5.0 min, purity = 99% (at 260 nm); λ_{max} (recorded during the HPLC analysis)/nm 258 and 382; LRMS (ESI-): *m/z* 320.1 $[M - H]^-$ (100) and 641.1 $[2M - H]^-$ (70), calcd for $C_{17}H_{11}N_3O_4$ 321.0; HRMS (ESI+): *m/z* 344.06339 $[M + Na]^+$, calcd for $C_{17}H_{11}N_3O_4Na^+$ 344.06418; HRMS (ESI-): *m/z* 320.06622 $[M - H]^-$, calcd for $C_{17}H_{10}N_3O_4^-$ 320.06713.

Dual PLE-NTR fluorogenic "turn-on" probe (5). Under Ar atmosphere, Ac-PHBA (60 mg, 0.36 mmol, 1 equiv.) was dissolved in dry DCM (3.6 mL). Diphosgene (60 µL, 0.504 mmol, 1. 4 equiv.) was then added and the resulting reaction mixture was cooled to 0 °C with an ice bath. Then DIEA (63 µL, 0.36 mmol, 1 equiv.) was added and the mixture was stirred again at 0 °C. Thereafter, the mixture was left to warm up to RT and stirred for a further 30 min. DCM was removed under vacuum. At the same time, a solution of phenol **3** (50 mg, 0.12 mmol, 0.33 equiv.) in dry THF (3 mL) was prepared and cooled to 0 °C under an Ar atmosphere. The previously prepared chloroformate derivative and TEA (47 µL, 0.35 mmol, 1 equiv.) were sequentially added and the reaction mixture was stirred at RT overnight. Thereafter, volatiles were removed under vacuum and the resulting residue was re-dissolved in DCM. The solution was filtrated to remove TEA.HCl and the filtrate was evaporated to dryness. The residue was purified by chromatography on a silica gel column (eluent: heptane-EtOAc, step gradient from 100 to to 70 : 30, v/v) to afford the fluorogenic "turn-on" probe **5** as yellow solid (23 mg, yield 32%, mixture of E and Z isomers). R_f 0.10 (DCM); ν_{max}(neat)/cm^{-1} 1754, 1595, 1523, 1433, 1375, 1258; δ_H(300 MHz, $CDCl_3$) 8.63 (s, 1 H), 8.38 (d, *J* 8.7, 1 H), 8.29 (d, *J* 8.7, 2 H), 8.08 (d, *J* 8.7, 1 H), 7.90 (d, *J* 7.2, 1 H), 7.64 (d, *J* 8.7, 2 H), 7.57-7.36 (m, 4 H), 7.14 (d, *J* 8.4, 2 H), 7.01 (dd, *J* 8.7, *J* 2.1, 1 H), 6.90 (d, *J* 2.1, 1 H), 5.29 (s, 2 H), 5.27 (s, 2 H), 2.32 (s, 3 H); δ_C(75 MHz, $CDCl_3$) 169.5, 157.0, 154.8, 154.3, 151.2, 143.7, 142.8, 140.2, 134.8, 132.0, 131.8, 130.0, 127.6, 127.0, 126.8, 126.6, 126.2, 124.0, 123.8, 122.0, 121.7, 119.9, 114.6, 114.0, 110.8, 105.9, 70.1, 69.7, 21.1; The complexity of the spectra (mixture of E and Z isomers, slow relaxation rate of some protons) for this compound makes a complete assignment impossible particularly for minor isomer. HPLC (system A): t_R = 5.9 and 6.2 min, purity = 94%; λ_{max} (recorded during the HPLC analysis)/nm 222 and 346 (weak, broad absorption band) for more polar isomer, 221, 364 and 372 for less polar isomer; LRMS (more polar isomer, ESI+): *m/z* 622.2 $[M + H]^+$ (100) and 429.3 $[M + H - C_{10}H_9O_4]^+$ (35, loss of *para*-acetoxybenzyloxycarbonyl moiety was occurred during the ionisation process); LRMS (more polar, isomer, ESI-): *m/z* 428.3 $[M - C_{10}H_9O_4]^-$ (100, loss of *para*-acetoxybenzyloxycarbonyl moiety was occurred during the ionisation process); LRMS (less

polar isomer, ESI+): *m/z* 622.1 $[M + H]^+$ (100) and 660.7 $[M + K]^+$ (25); LRMS (less polar isomer, ESI-): *m/z* 428.3 $[M - C_{10}H_9O_4^+]^-$ (100, loss of *para*-acetoxybenzyloxycarbonyl moiety was occurred during the ionisation process), calcd for $C_{33}H_{23}N_3O_8S$ 621.1; HRMS (ESI+): *m/z* 622.12739 $[M + H]^+$, calcd for $C_{33}H_{24}N_3O_8S^+$ 622.12786 and 644.10859 $[M + Na]^+$, calcd for $C_{33}H_{23}N_3O_8SNa^+$ 644.10981.

Dual PLE-NTR fluorogenic "turn-on" probe (8).

(a) Preparation of para-nitrophenyl para-acetoxybenzyl carbonate[34]*:* Ac-PHBA (200 mg, 1.20 mmol, 1 equiv.), TEA (0.325 mL, 2.41 mmol, 2 equiv.) were dissolved in dry THF. *para*-Nitrophenyl chloroformate (485 mg, 2.41 mmol, 2 equiv.) was added, and the resulting reaction mixture was stirred under Ar atmosphere for 90 min. Thereafter, EtOAc (50 mL) was added and the organic medium was washed with aq. 1.0 M HCl (3 × 10 mL), aq. saturated $NaHCO_3$ (3 × 15 mL) and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure and the resulting crude product was purified by chromatography on a silica gel column (eluent: DCM-EtOAc-TEA 50 : 10 : 1.5, v/v/v). The activated carbonate **6** was obtained as white solid (228 mg, yield 57%). δ_H(300 MHz, $CDCl_3$) 8.30 (d, *J* 9.3, 2 H), 7.48 (d, *J* 8.7, 2 H), 7,40 (d, *J* 9, 2 H), 7.16 (d, *J* 8.7, 2 H), 5.30 (s, 2 H), 2,33 (s, 3 H).

(b) Phenol protection: Under Ar atmosphere, a solution of activated carbonate **6** (377 mg, 1.14 mmol, 3 equiv.) in dry THF (4.2 mL) was added to phenol **4** (122 mg, 0.38 mmol, 1 equiv.). Then TEA (153 µL, 1.14 mmol, 3 equiv.) and DMAP (catalytic amount) were sequentially added. The resulting reaction mixture was stirred at RT for 1 h. Thereafter, solvent was removed under vacuum and the resulting residue was taken with DCM and washed with aq. 0.1 M K_2CO_3 (2 × 10 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by chromatography on a silica gel column (eluent: heptane-EtOAc, step gradient from 100 to 70 : 30, v/v) to give the desired fluorogenic "turn-on" probe as light yellow solid (60 mg, yield 30%). R_f 0.30 (DCM); ν_{max}(neat)/cm^{-1} 2956, 2924, 2228, 1762, 1604, 1589, 1518, 1435, 1357, 1245; δ_H(300 MHz, $CDCl_3$) 8.31-8.26 (m, 3 H), 8.20 (s, 1 H), 7.57 (d, *J* 8.7, 2 H), 7.45 (d, *J* 8.7, 2 H), 7.14 (d, *J* 8.7, 2 H), 6.53 (dd, *J* 9.0, *J* 2.4, 1 H), 6.93 (d, *J* 2.4, 1 H), 5.26-5.24 (2s, 4 H), 2.31 (s, 3 H); δ_C(75 MHz, $CDCl_3$) 169.3, 158.4, 156.8, 152.5, 152.2, 151.2, 148.2, 141.7, 131.7, 130.3, 130.1, 128.2, 124.3, 122.1, 118.2, 114.8, 114.0, 112.7, 105.9, 82.3, 70.3, 70.1, 21.1; HPLC (system A): t_R = 5.7 min, purity = 94% (at 260 nm); λ_{max} (recorded during the HPLC analysis)/nm 296 and 356; LRMS (ESI-): *m/z* 512.3 $[M - H]^-$ (10) and 320.2 $[M - C_{10}H_9O_4]^-$ (100, loss of *para*-acetoxybenzyloxycarbonyl moiety was occurred during the ionisation process), calcd for $C_{27}H_{19}N_3O_8$ 513.1; HRMS (ESI+): *m/z* 536.10566 $[M + Na]^+$, calcd for $C_{27}H_{19}N_3O_8Na^+$ 536.10644.

Dual PGA-NTR fluorogenic "turn-on" probe (9).

(a) Preparation of para-nitrophenyl para-(phenylacetamido)benzyl carbonate[35]*:* PhAc-PABA (200 mg, 0.829 mmol, 1 equiv.), TEA (0.223 mL, 1.66 mmol, 2 equiv.) and DMAP (catalytic amount) were dissolved in dry THF. *para*-Nitrophenyl chloroformate (334 mg, 1.66 mmol, 2 equiv.) was added and the resulting reaction mixture was stirred under an Ar atmosphere for 30 min. Thereafter, EtOAc (50 mL) was added and the organic medium was washed with aq. 1.0 M HCl (3 × 10 mL), aq. saturated $NaHCO_3$ (3 × 15 mL) and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure and the resulting crude product was purified by chromatography on a silica gel column (eluent: DCM-EtOAc-TEA 50 : 10 : 1.5, v/v/v). The activated carbonate **7** was obtained as white solid (194 mg, yield 58%). δ_H(300 MHz, $CDCl_3$) 8.26 (d, *J* 9.3, 2 H), 7.50 - 7.34 (m, 11 H), 7.12 (s, 1 H, N*H*), 5.22 (s, 2 H), 3.76 (s, 2 H).

(b) Phenol protection: Under an Ar atmosphere, a solution of activated carbonate **7** (194 mg, 0.48 mmol, 1.2 equiv.) in dry THF (4 mL) was added to phenol **4** (128 mg, 0.398 mmol). Then TEA (54 µL, 0.40 mmol, 1 equiv.) and DMAP (catalytic amount) were sequentially added. The resulting reaction mixture was stirred at RT for 1 h. Thereafter, solvent was removed under vacuum and the resulting residue was taken with DCM and washed with aq. 0. 1 M K_2CO_3 (2 × 10 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The residue was firstly purified by chromatography on a silica gel column (eluent: heptane-EtOAc, step gradient from 100 to 70 : 30, v/v) and then by crystallization in pre-cooled (0 °C) mixture EtOH-acetone (8 : 2, v/v) to obtain yellow solid (40 mg, yield 17%). R_f 0.84 (DCM-EtOAc, 9: 1, v/v); ν_{max}(neat)/cm^{-1} 3289, 3064, 2229, 1767, 1657, 1605, 1521, 1361, 1229; δ_H(300 MHz, DMSO-d_6) 10.25 (s, 1 H), 8.55 (s, 1 H), 8.28 (d, *J* 8.4, 2 H), 8.07 (d, *J* 8.7, 1 H), 7.80 (d, *J* 8.7, 2 H), 7.64 (d, *J* 8.7, 2 H), 7.41-7.12 (m, 9 H), 5.42 (s, 2 H), 5.22 (s, 2 H), 2.65(s, 2 H); δ_C(75 MHz, DMSO-d_6) 169.7, 158.8, 156.6, 155.5, 152.4, 147.8, 143.9, 140.1, 136.4, 130.6, 129.9, 129.7, 129.6, 129.2, 128.8, 127.0, 124.1, 119.5, 118.8, 115.2, 114.7, 113.7, 107.8, 82.7, 70.7, 79.9, 43.8; HPLC (system A): t_R = 5.7 min, purity = 94% (at 260 nm); λ_{max} (recorded during the HPLC analysis)/nm 231, 295 and 356; LRMS (ESI-): *m/z* 320.1 $[M - C_{16}H_{14}NO_3]^-$ (100, loss of *para*-phenylacetamidobenzyloxycarbonyl moiety was occurred during the ionisation process); LRMS (ESI+): *m/z* 589.2 $[M + H]^+$ (100), calcd for $C_{33}H_{24}N_4O_7$ 588.1; HRMS (ESI+): *m/z* 589.17148 $[M + H]^+$, calcd for $C_{33}H_{25}N_4O_7^+$ 589.17178 and 611.15320 $[M + Na]^+$, calcd for $C_{33}H_{24}N_4O_7Na^+$ 611.15372.

***In vitro* activation of fluorogenic "turn-on" probes 5, 8 and 9 by hydrolase (PGA or PLE) and reductase (NTR) - Fluorescence assays and HPLC-MS analyses**

See ESI† for experimental details.

Abbreviations (not defined within the text)

BZT	2-benzothiazolyl
calcd	calculated
CBT	2-cyanobenzothiazole
CRABPII	protein reengineering of cellular retinoic acid binding protein II
DCM	dichloromethane

DMAP	4-*N*,*N*-dimethylaminopyridine
EtOH	ethanol
Et_2O	diethyl ether
FA	formic acid
FRET	fluorescence resonance energy transfer
HPLC	high-pressure liquid chromatography
MS	mass spectrometry
NADH	nicotinamide adenine nucleotide (reduced form)
PE	petroleum ether (bp 40-60 °C)
rt	room temperature
TBAF	tetrabutylammonium fluoride
TBDMS	*tert*-butyldimethylsilyl
TBDMS-Cl	*tert*-butyldimethylsilyl chloride
TEA	triethylamine
THF	tetrahydrofuran
UV-vis	ultraviolet-visible

Acknowledgements

Financial support from Institut Universitaire de France (IUF) and the Burgundy region ("FABER" programme, PARI Action 6, SSTIC 6 "Imagerie, instrumentation, chimie et applications biomédicales"), especially for the Ph. D. grant of S. D., is greatly acknowledged. The authors thank the "Plateforme d'Analyse Chimique et de Synthèse Moléculaire de l'Université de Bourgogne" (PACSMUB, http://www.wpcm.fr) for the access to HORIBA Jobin Yvon Fluorolog spectrofluorimeter, Thermo LTQ Orbitrap XL mass spectrometer, Bruker Avance III 300 and 500 spectrometers and Bruker Alpha FT-IR spectrometer. The authors also thank Marie-José Penouilh (IE, University of Burgundy, ICMUB-WPCM) and Dr. Fanny Picquet (IE, ICMUB-WPCM) for recording the HRMS spectra, and Benoît Roubinet (Ph. D. student, University of Rouen, laboratory COBRA UMR CNRS 6014) for the synthesis of 3-(2-benzothiazolyl)-7-hydroxy-(2-imino)coumarins used as references in this work.

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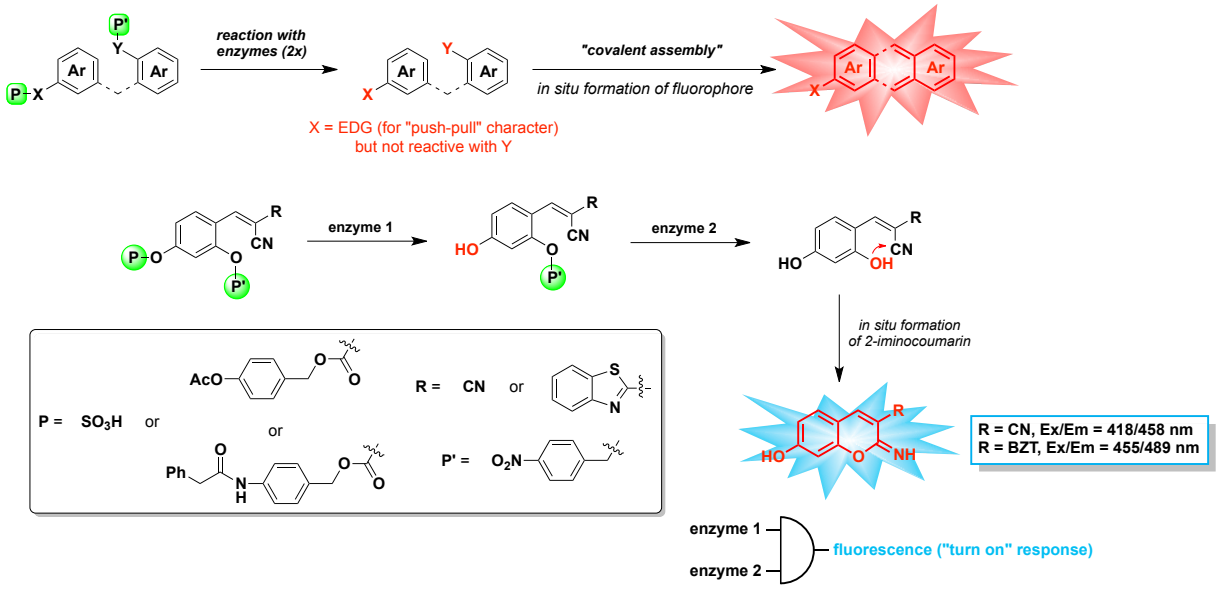
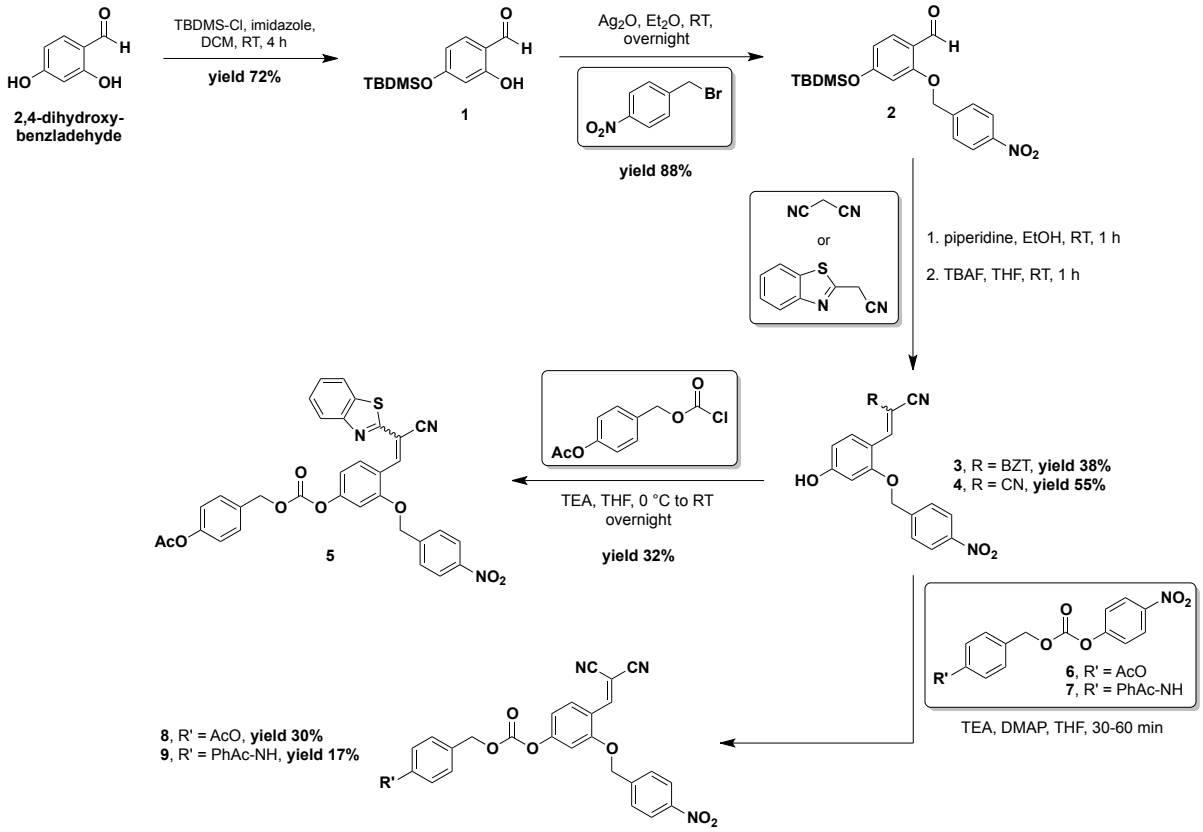


Fig. 1 Strategy explored in this work: (top) "covalent assembly" principle applied to the simultaneous detection of two distinct enzymes (X = NH or O, EDG = electron-donating group); (bottom) *in situ* synthesis of 3-substituted-7-hydroxy-2-iminocoumarin scaffolds from a non-fluorescent caged precursor and through domino reactions triggered by the two different enzymes (failed attempt: probe for sulfatase-NTR, R = CN, P = SO_3H and P' = *para*-nitrobenzyl; probes for PLE-NTR, R = BZT or CN, P = Ac-PHBoxycarbonyl and P' = *para*-nitrobenzyl; probe for PGA-NTR, R = CN, P = PhAc-PABoxycarbonyl and P' = *para*-nitrobenzyl). BZT = 2-benzothiazolyl, NTR = nitroreductase, PLE = pig liver esterase and PGA = penicillin G acylase.



Scheme 1 Synthesis of dual-enzyme fluorogenic "turn-on" probes **5**, **8** and **9**. *Please note*: molecule corresponding to chloroformate derivative of Ac-PHBA is not numbered because not isolated and directly used without purification.

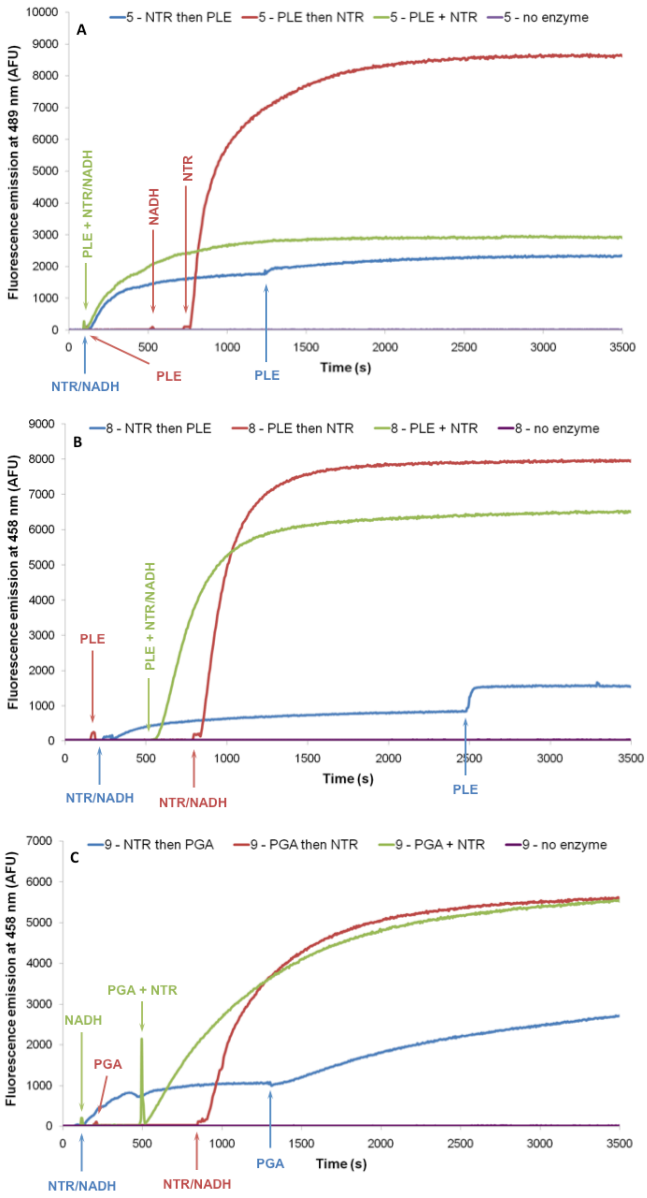


Fig. 2 Time-dependant changes in the blue/green fluorescence intensity of fluorogenic "turn-on" probes **5**, **8** and **9** (concentration: 1.0 µM) in the presence of two distinct enzymes in PB at 37 °C (see ESI† for more details about sequential and simultaneous incubations protocols with NTR (0.1 U) / NADH (45 µM), PLE (1 U) and PGA (1 U)). (A) Ex./Em. 455/489 nm; (B)-(C) Ex./Em. 418/458 nm. For each probe, a further kinetic curve (purple trace) was recorded under the same conditions but without enzymes and co-factor.

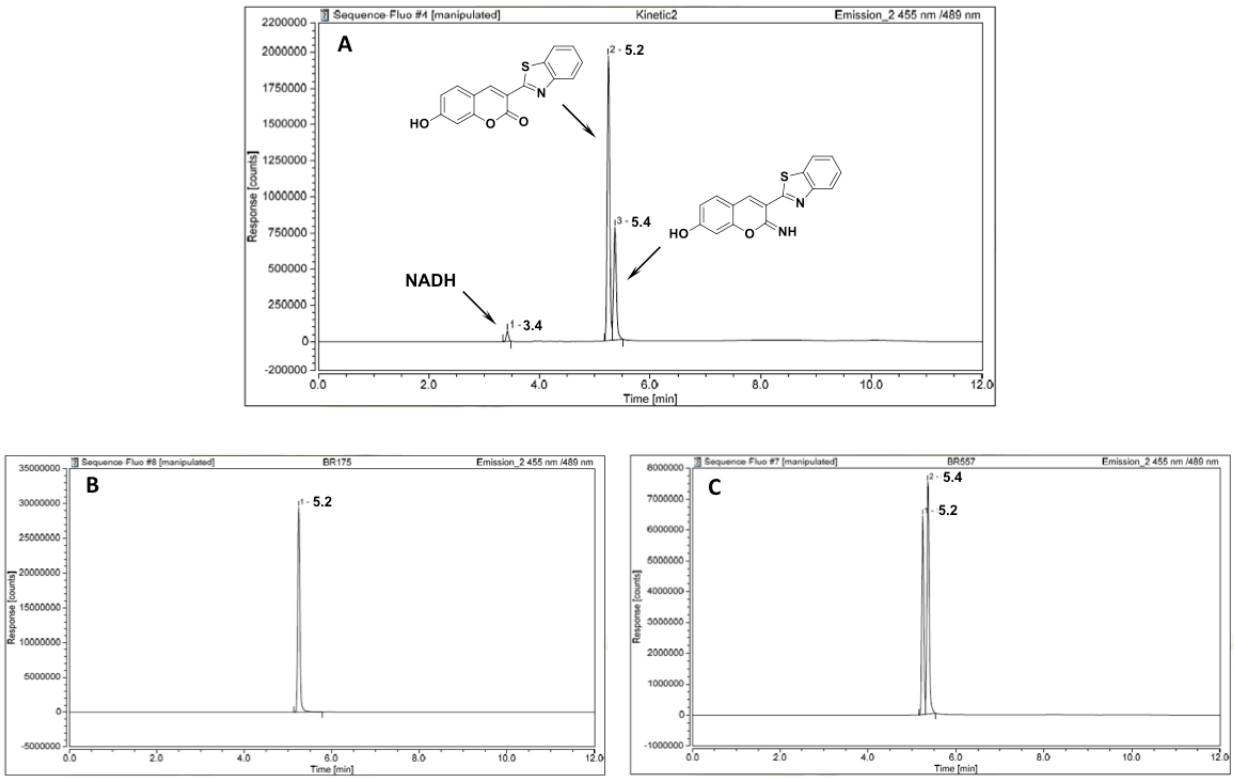


Fig. 3 RP-HPLC elution profiles (fluorescence detection, system E) of enzymatic reaction mixture of benzothiazolyl-based probe **5** with PLE (10 min of pre-incubation) and NTR/NADH (A) and authentic samples of 3-(2-benzothiazolyl)-7-hydroxycoumarin (B) and 3-(2-benzothiazolyl)-7-hydroxy-2-iminocoumarin (C). *Please note*: partial hydrolysis of imine moiety was occurred during HPLC analysis and incubation in PB. NADH (t_R = 3.4 min) can be properly detected at a different wavelength channel (Ex./Em. 350/460 nm).

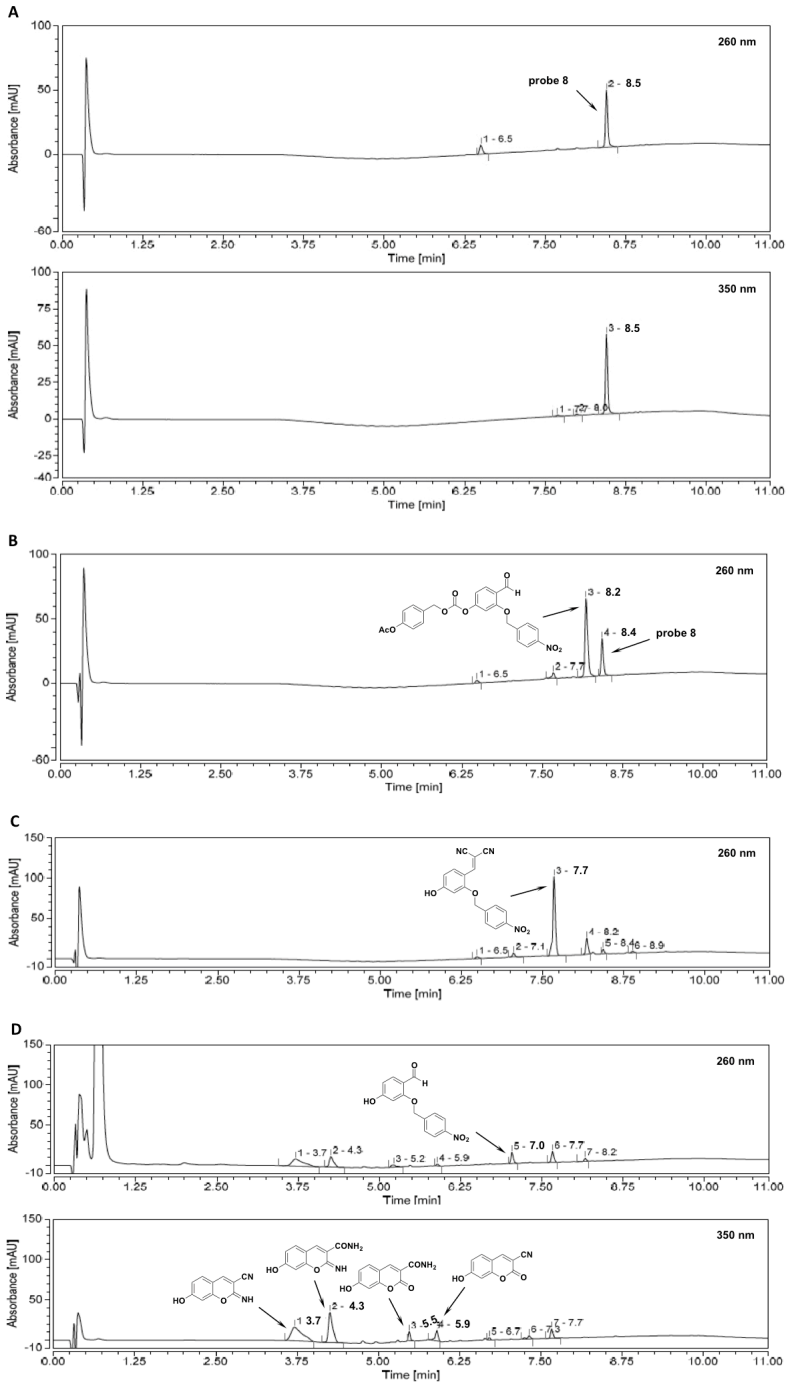
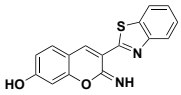
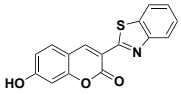
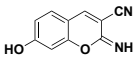
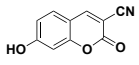
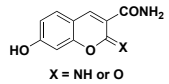
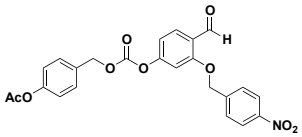
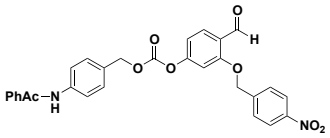
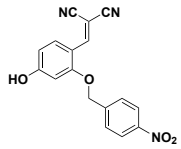
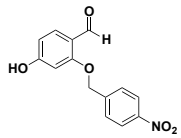


Fig. 4 RP-HPLC elution profiles (UV-vis and MS detection, system C) of cyano-based probe **8**: (A) before incubation in PB; (B) after 10 min of incubation in PB at 37 °C (retro-Knoevenagel product was not detected at 350 nm); (C)-(D) after dual-enzymatic activation at 37 °C (sequential protocol, see ESI† for experimental details): (C) after 30 min of incubation with PLE (0.55 U) (compound **4** was not detected at 350 nm); (D) after 80 min of incubation with PLE (0.55 U) alone and further 40 min with NTR/NADH (0.6 U/0.58 mM). See figure S7 for all ESI mass spectra. See figures S8-S9 for all RP-HPLC elution profiles related to dual-enzymatic activation of probe **8** (sequential "NTR/NADH then PLE" and simultaneous incubation protocols) and probe **9** (sequential "PGA then NTR/NADH" and "NTR/NADH then PGA" and simultaneous incubation protocols).

Table 1 RP-HPLC retention times, LR-MS data and/or spectral properties of compounds formed through the dual-enzyme activation of probes **5**, **8** and **9**.

Compound	RP-HPLC-fluorescence t_R (min)[a]	RP-HPLC-MS t_R (min)[b]	Molecular ion detected	Molecular ion calcd	Abs./Em. (nm)	Φ_F (%)
	5.4	-	-	-	446/493[c]	43[c]
	5.2	-	-	-	431/488[d]	57[d]
	3.9	3.7	187.5 $[M + H]^+$	187.2 $[M + H]^+$	418/458[e]	37[f]
	4.1	5.9	186.4 $[M - H]^-$	186.1 $[M - H]^-$	409/452[e]	85[f]
	3.6	4.3 (X = NH) 5.5 (X = O)	203.2 $[M - H]^-$ 204.4 $[M - H]^-$	203.2 $[M - H]^-$ 204.2 $[M - H]^-$	-	-
	-	8.2	510.1 $[M - H + FA]^-$	510.4 $[M - H + FA]^-$	-	-
	-	8.3	584.9 $[M - H + FA]^-$	585.5 $[M - H + FA]^-$	-	-
	-	7.7	320.3 $[M - H]^-$	320.3 $[M - H]^-$	-	-
	-	7.0	272.3 $[M - H]^-$	272.2 $[M - H]^-$	-	-

[a] system D. [b] system C. [c] see ref. 45. [d] see ref. 21. [e] determined in PB at 25 °C. [f] determined in PB at 25 °C using quinine sulfate (Φ_F = 60% in 0.05 M H_2SO_4) as a standard.