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An Autoimmolative Spacer Allows First-Time Incorporation of a Unique Solid-State Fluorophore into a Detection Probe for Acyl Hydrolases

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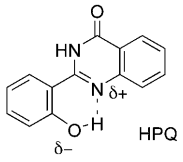
The challenges and the promises for the field of advanced chemical imaging have recently been assessed by a group of experts.[1] The issues concerning optical modalities, and especially fluorescence-based techniques, have been identified in "the need for greater spatial control" and in "greater robustness of fluorescent probes". Such fluorogenic imaging probes would be equally attractive for applications in vitro and in vivo. Current probes are mostly based on water-soluble fluorophores; probes that have previously found extensive use in in vitro assays of purified proteins without any need for spatial distinction. Localization of specific molecular entities in biological tissue is mostly done with the aid of antibodies conjugated to a fluorophore (immunohistochemistry). Detecting a particular biochemical property in an in situ context in the life sciences is always associated with the kinetics of two processes: 1) diffusion of the probe toward (and away from) the site where the activity is present and 2) the kinetics of signal generation—be it complex formation or enzymatic modification. These effects may more or less cancel each other out and no particular choice of incubation or readout time for the assay will give meaningful imaging results.

We have chosen a fluorophore, 2-(2′-hydroxyphenyl)-4(3*H*)-quinazolinone (HPQ), that is completely insoluble in aqueous media, but strongly fluorescent in the solid state (Figure S1 in the Supporting Information). Fluorescent dyes that exhibit excited-state intramolecular proton transfer (ESIPT) have attracted great interest for several decades because such compounds show good photophysical properties, such as intense luminescence, large Stokes shifts, and significant photostability.[2–4] HPQ thus distinguishes itself greatly from common fluorescent dyes, such as water-soluble fluorescein, which exhibits negligible emission in the solid state due to a small Stokes shift and consequent self-quenching (Figure S1 in the Supporting Information). The large Stokes shift of HPQ contributes two additional, very welcome advantages: an increase in sensitivity and minimization of background fluorescence. Both its strong fluorescence and insolubility are intimately associated with the internal hydrogen bond between the phenolic hydrogen and the imine nitrogen. Should one hinder this interaction by attaching a polar, enzyme-susceptible group to this oxygen atom, then a water-soluble imaging probe would be formed that has the long-wavelength fluorescence of HPQ efficiently eliminated. It does not come as a surprise that probes for particular enzymes (β-glucuronidase and alkaline phosphatase) have been reported and commercialized.[5–8] However, no functioning HPQ-based probe has ever been introduced for the assaying or imaging of the huge classes of peptidases and lipases. Both classes comprise prominent biomarkers for various diseases, play an important role in proteome profiling, and are also subjected to extensive modification by directed evolution, which requires enormously efficient tests, preferably in the bacterial colony assay format, in view of the large libraries to be screened. We report herein, a molecule that effectively detects a model peptidase, a molecule that shows elevated robustness, and that should allow for the adaptation to diffusion-resistant, sequence-specific detection of peptidases for maximal spatial control.[9]



Many enzyme-responsive probes have been designed in which the fluorophore is a phenol or an aniline that is directly attached to the scissile bond.[10,11] Importantly, phenolic esters, in particular, are prone to spontaneous hydrolysis (background signal generation). Also, we have recently shown that an aniline-type derivative of HPQ does not show any appreciable fluorescence.[12] HPQ esters have an even higher tendency for spontaneous hydrolysis

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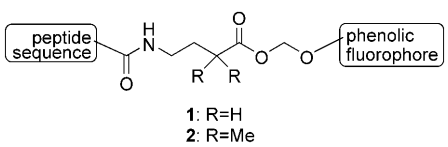
than regular phenol esters, which we have observed ourselves.[13] Previously, others have postulated that a self-catalytic effect (anchimeric assistance by the imine) is responsible for this property while studying a different, albeit related, molecular system.[14] We were thus forced to come up with a substituent other than acyl on the phenolic oxygen of HPQ. Acylated O,O-acetals have been used for a long time in the design of prodrugs of phosphorylated species to render them electroneutral for better bioavailability.[15,16] More recently, they were employed to devise detection probes incorporating soluble phenolic fluorophores.[17–19] Here, they still showed a residual background hydrolysis, except for a pivaloyl O,O-acetal group, which was completely immune to it.[20] We thus selected this solution in our target design.

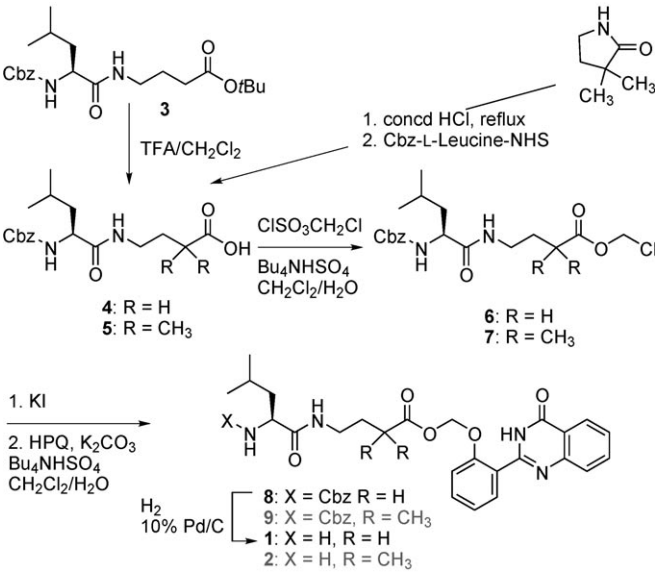
Communicating the event of peptide-bond cleavage rapidly to the part of the molecule that can liberate the fluorophore requires employment of a self-immolative linker. Such linkers (also known as traceless linkers in solid-phase organic synthesis) have found wide-spread application in the field of prodrug activation,[21] since the first such spacers were introduced in the early 1980s.[22] In recent years there has been increasing interest in the design of three-component enzyme substrates (trigger–linker–fluorophore) by using self-immolative spacers as linkers.[23] Such three-component substrates display unique selectivity and limited interference with environmental effects because these systems unmask their intense fluorescence only by a specific enzymatic cleavage.[24–31] According to the mechanism of active-compound release, the linkers can be classified as cyclization or elimination linkers.[21] One of the first and most frequently employed self-immolative spacers is *p*-aminobenzyloxycarbonyl (PABC). Very recently, this linker has been employed in the design of two three-component substrates for proteases containing soluble phenolic fluorophores (e.g., 7-hydroxycoumarine) to circumvent the above-mentioned problems of two-component systems.[31,32]

Taking into account our acylated O,O-acetal moiety, we opted instead for a cyclization linker that immolates via an energetically favorable five- or six-membered transition state, thus setting a limit to the distance of the fluorogenic group and the scissile carboxamide bond. We identified the synthetically more accessible targets **1** and **S13** (Scheme S1 in the Supporting Information) that contain a γ-aminobutyric acid (GABA) derived linker and target **2**, which features a 2,2-dimethyl GABA unit. Compound **2** can be predicted to immolate more rapidly due to the Thorpe–Ingold effect.[33]

To test whether a GABA-based three-component system fragments reliably, we first synthesized a GABA model compound (**S13**, Scheme S1 in the Supporting Information) with a carbobenzyloxy (Cbz) group on its amino terminus, instead of a peptidase substrate moiety, and mimicked the action of an enzyme by hydrogenative removal of this Cbz group. When **S13** was hydrogenated with 5% Pd/C in MeOH under ambient pressure and temperature for 1 h, TLC analysis indicated the release of HPQ to be complete. By using acidic conditions (10% acetic acid in MeOH), the presence of the nonimmolated intermediate **S14** on a TLC plate could be confirmed under a UV lamp (365 nm) because it can be easily distinguished from the strongly fluorescent HPQ. However, even evaporation of the sample without any heating did not allow us to isolate **S14**, which simply fragmented too easily (Scheme S1 in the Supporting Information).

With these encouraging results we equipped our two related linkers with a leucine residue to test our probe candidates in a real-world situation by using microsomal leucine aminopeptidase (EC 3.4.11.2) as an efficient model enzyme (Scheme 1). Thus, target **1** was synthesized by preparing **3** from commercial starting materials by a simple peptide coupling reaction. The free acid **4**, thus obtained, had to be transformed into the chloromethyl ester. The classic procedure[34] of treatment of the free acid with paraformaldehyde and $ZnCl_2$ in dry toluene did not result in the formation of any **6**, even when the reaction was heated to 55 °C for 24 h, as determined by TLC and LC–MS analysis. A more recent strategy,[35] carried out by using the commercial reagent chloromethyl chlorosulfate and phase-transfer conditions gave **6** in excellent yield (88%) in only 1 h. Iodination prior to coupling with a phenol has been reported to be superior to direct coupling of the chloride. Several strategies were





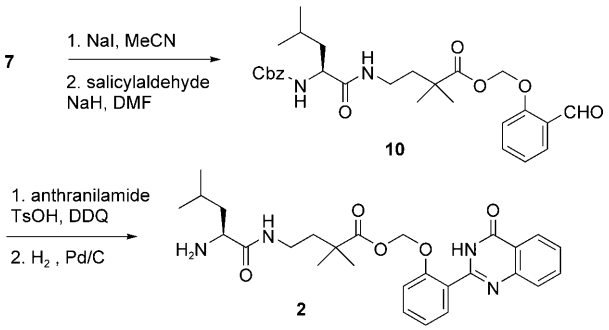
Scheme 1. Synthetic strategy to access target compounds **1** and **2** by employing phase-transfer conditions. Instead of **9**, a product of identical mass was formed from a coupling with the N–H site of HPQ; TFA = trifluoroacetic acid, OSu = succinate ester.

described on how to couple iodomethyl esters with a phenol: standard single-phase conditions with acetonitrile or acetone as the solvent and K_2CO_3 as the base at room temperature,[18] strong single-phase conditions with DMF/THF as the solvent and NaH as the base at low temperature,[17] and a biphasic system in the presence of tetrabutylammonium hydrogen sulfate as the phase-transfer catalyst.[35] We were worried about interference of the N–H acidic site on HPQ and opted for the mildest procedure, namely, that employing biphasic conditions. This gave **8** in low yield, the hydrogenation of which necessitated the use of 10% Pd/C, but then gave **1** in quantitative yield in 2 h.

Target **2**, containing two methyl groups on the α carbon of the linker, required us to exhaustively methylate pyrrolidin-2-one according to a literature procedure (Scheme 1).[36] The resulting α,α-dimethyl-γ-lactam can be opened by heating in concentrated hydrochloric acid. The strong tendency of this GABA derivative to recyclize makes a special procedure necessary, that is, the activation of Cbz-Leucine as a succinate ester and its smooth conversion with the obtained hydrochloride in the presence of triethylamine to give the free acid **5** in 57% yield. Although this acid can be easily transformed into **7** with the established biphasic conditions, the corresponding iodide of **7** could not be coupled with HPQ, even though we tried all of the strategies described above. In fact, TLC and mass analysis indicated the exclusive formation of a compound resulting from coupling of the N–H site of HPQ. This compound was less polar and possessed the same mass as **9**. It emitted a green fluorescence under a UV lamp (365 nm) because the phenolic hydroxyl was still intact. In contrast, compound **9** was more polar than HPQ itself and showed absolutely no fluorescence under a UV lamp (365 nm). Similar byproducts were also found in the synthesis of **8**.

To avoid this unwelcome complication, we turned to another strategy, which was to introduce the HPQ unit only upon completion of the trigger–linker–phenol unit. Coupling of the iodide of **7** with salicylaldehyde thus furnished aldehyde **10** by employing monophasic conditions and a strong base to overcome the weak acidity of salicylaldehyde, which exhibits an internal hydrogen bond. Condensation of this aldehyde with anthranilamide, followed by oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and catalytic hydrogenation gave target **2** without any byproducts and in satisfactory yield (65%; Scheme 2).

The photophysical properties of the proposed fluorogenic probes **1** and **2** were evaluated under simulated physiological conditions (Tris-HCl, pH 7.4, Tris = tris(hydroxymethyl)aminomethane). As predicted, profluorescent substrates **1** and **2** emitted no fluorescence at 495 nm (maximum emission wavelength for free HPQ). However, after incubation of **2** with leucyl aminopeptidase for 1 h, intense fluorescence at 495 nm was observed (Figure 1). This initial result supported the conclusion that **2** is an effective substrate for the enzyme and releases insoluble HPQ. The kinetics of the transformation of **1** and **2** were followed with a spectrofluorimeter (Figure 2). The maximum fluorescence intensity



Scheme 2. Isomer-free access to substrate **2**; TsOH = *para*-toluene sulfonic acid.

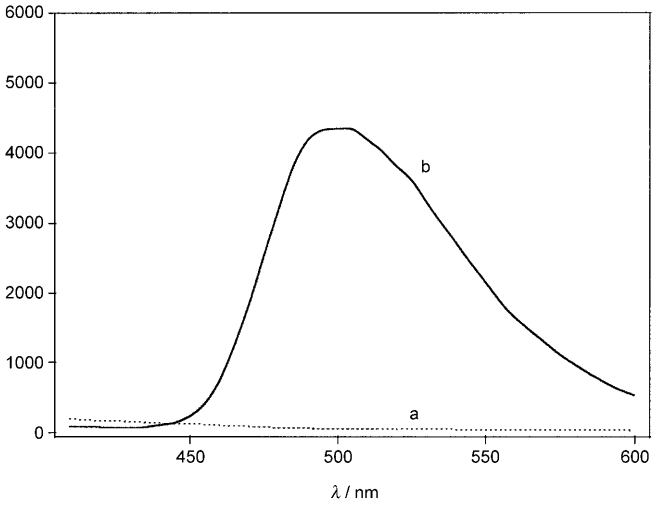


Figure 1. Emission spectra (λ_{Ex} = 365 nm) of probe **2** (20 μM) before (a) and after (b) incubation with leucyl aminopeptidase for 1 h at 25 °C in Tris-HCl.

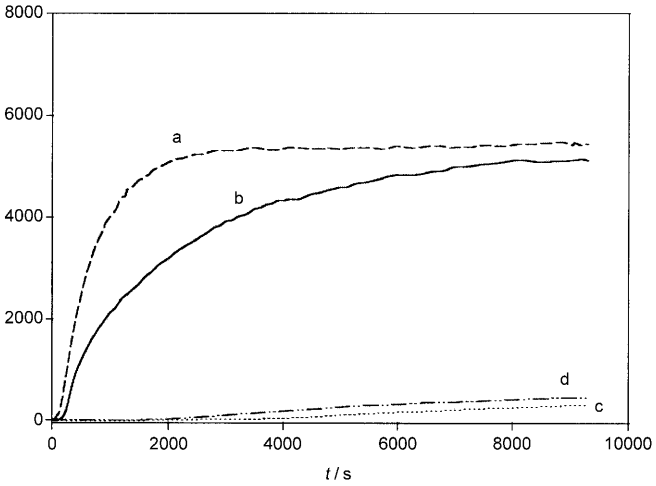


Figure 2. Fluorescence assay (λ_{Ex} = 365 nm, λ_{Em} = 495 nm) for the cleavage of substrates **1** and **2** with leucyl aminopeptidase in Tris-HCl (25 mM, pH 7.4), substrate (38 μM), leucyl aminopeptidase (0.02 U mL^{-1}), 25 °C. With enzyme: a) substrate **2**, b) substrate **1**. Without enzyme (bovine serum albumin (0.1 mg mL^{-1}) was added): c) substrate **2**, d) substrate **1**.

attests to the quantitative conversion of **1** and **2** into free HPQ. As expected, the highly preorganized spacer in **2** led to a superior rate of conversion with the maximum intensity reached after about 35 min at the indicated assay conditions. But substrate **1** also showed acceptable kinetics with the maximum fluorescence reached after about 150 min. More importantly, substrate **2** showed a lower background in signal generation (Figure 2, curve c) than that of substrate **1** (Figure 2, curve d), in congruence with the reported behavior for a pivaloyl O,O-acetal.[20] The fact that a residual background can still be observed for **2** may be explained by the presence of the free N terminus. The observed kinetic parameters (apparent Michaelis constant, $K_{M,app}$ and apparent maximal reaction rate, $V_{max,app}$) for probe **2** with leucyl aminopeptidase at 25 °C were determined as $K_{M,app} = 98\ \mu M$ and $V_{max,app} = 5.12 \times 10^{-8}\ M\,s^{-1}$ (25 mM Tris-HCl, pH 7.4, 25 °C). The $K_{M,app}$ value is in the same range as that of Leu-7-amido-4-methylcoumarin ($K_M = 39.6\ \mu M$) at comparable assay conditions,[37] whereas its $V_{max,app}$ value is three orders of magnitude larger than that of the latter ($V_{max} = 7.2 \times 10^{-11}\ M\,s^{-1}$).

In conclusion, we have been able to employ the unique solid-state fluorophore HPQ in the design of a water-soluble nonfluorescent probe for a particular peptidase. The synthesis developed opens up the possibility to prepare a large variety of probes for *exo*-(amino)peptidases, but we do not rule out the possibility that sequences may be found that include modifications of the GABA spacer to make the probe susceptible to selected endoproteases as well. Many applications in the life sciences (e.g., high-throughput screening as well as in vivo imaging) can be envisaged in which such probes assay or image peptidases in a true off/on mode without suffering from diffusion-related signal dilution.

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