A comparative study of the self-immolation of *para*-aminobenzylalcohol and hemithioaminal-based linkers in the context of protease-sensitive fluorogenic probes†

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This study focuses on the disassembly-behavior of self-immolative pro-fluorescent linkers under physiological conditions and through an enzyme-initiated domino reaction. The targeted linkers are based on *para*-aminobenzylalcohol (PABA) or hemithioaminal derivatives of *para*-carboxybenzaldehyde or glyoxilic acid. We found that a fine tuning of the kinetic properties could be obtained through the modulation of the linker structure, giving either a fast signal response or free-adaptable systems suitable for the design of protease-sensitive fluorogenic probes or prodrug systems.

The design and optimisation of new enzymatically triggered self-immolative linkers take an even more critical place in the development of efficient prodrug systems[1] and smart optical bioprobes suitable for bioanalytical and biological applications such as high-throughput screening or biomolecular imaging.[2] Indeed, in such molecular constructions, self-immolative linkers are key components to control the release of the free active product (*i.e.*, drug or optical marker) upon designed (enzymatic, chemical or physical) activation under mild neutral and aq. conditions. For instance, in the emerging and promising field of pro-fluorescent bioprobes, the targeted tripartate sensor (*i.e.*, a molecular probe composed of a trigger recognition unit, a linker and a fluorophore moiety) unmasks its intense fluorescence only by a specific enzymatic cleavage that produces a labile self-immolative linker-fluorophore intermediate that in turn eliminates spontaneously to release the original fluorescent marker through a domino reaction[3–5] (see Fig. 1 for the application of this concept to proteases).

These latent fluorescent probes thus display a unique selectivity and limited interferences associated with the probe concentration, excitation intensity, and emission sensitivity.[6] Moreover, the use of such linkers is potentially beneficial to improve their chemical stability and enzymatic reactivity by moving away the bulky hydrophobic fluorophore from the enzyme recognition unit.[4,5] Such a self-immolative linker strategy also widens the scope of the available pro-fluorophores (*i.e.*, aniline-, phenol- or thiophenol-based fluorophores) for a targeted enzymatic reaction. For example, as illustrated by us, it allows the development of efficient remote protease-coupled fluorescent phenol release systems.[5,7]

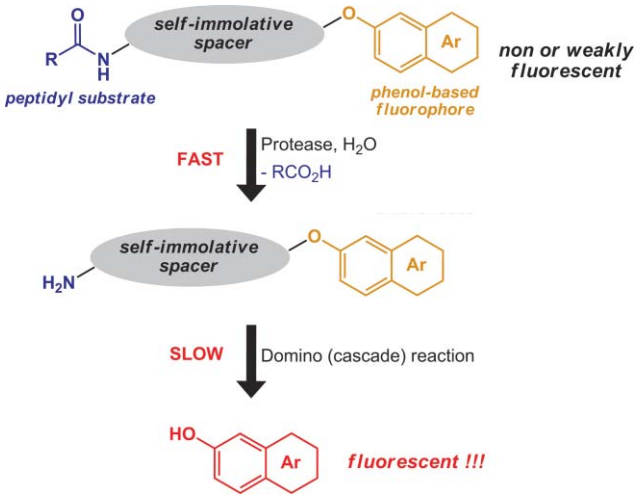

Fig. 1 General principle for the activation of protease-sensitive fluorogenic probes based on a self-immolative linker strategy.

To perform real-time biomolecular imaging, the release of the latent fluorophore after target recognition should be as fast as possible in order to accurately track the biological event. However during self-immolation, the domino (or cascade) reaction is often slower than the enzyme activation step due to the occurrence of electronic rearrangement or cyclisation processes which may induce a significant delay time between the enzyme cleavage and the observation of a significant emission of the free excited fluorophore.

During the past decade, significant research efforts have been devoted to the development of sophisticated prodrug technologies involving drug delivery vehicles with self-eliminating and multiple release properties.[1,8] Since the most commonly used self-eliminating structures are based on *para*-aminobenzylalcohol (PABA) linker (or its hydroxylated analogue),[9] some academic groups have recently reported comprehensive kinetic studies for the disassembly of such self-immolative systems through 1,4- and/or 1,6-benzyl elimination reactions. Thus, Shabat *et al.* have

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published an elegant and sophisticated molecular system that can disassemble through *para*- and *ortho*-azaquinone-methide elimination reactions in which the 1,6-elimination process was found to be faster than the 1,4-elimination one.[10] Similarly, Shabat *et al.* have shown that the pyridinone-methide elimination was even faster than the parent azaquinone-methide process under physiological conditions.[11] Interestingly, Lee *et al.* have also reported a kinetic study on self-immolation reactions concerning the 1,4- and 1,6-elimination reactions of quinone-methide systems showing an efficient "shortcut" that outperforms conventional pathways involving repetitive quinone-methide rearrangements and elimination.[12] In the context of protease-sensitive fluorogenic probes, we have explored three different self-immolative spacer strategies based either: (1) on the use of an intramolecular lactam-cyclisation process with the enzyme-labile safety catch linker reported by Waldmann *et al.*[13] (Fig. 2), (2) on the use of the self-cleavable PABA linker or (3) on the design of an original hemithioaminal core.[14] The resulting pro-fluorophores are able to release fluorescent umbelliferone (or its 4-acetic acid derivative) within few minutes in the presence of the model protease penicillin G acylase (PGA) under physiological conditions. However, in the first case, the limited stability of the aryl ester moiety prevented its generalised use for the latent fluorescent probes design.[15] In the third case, a slow linear increase in fluorescence intensity was observed without the enzyme, corresponding to the non-enzymatic hydrolysis of its carbonate moiety (compound **1**, Fig. 3a). To improve its stability, the carbonate linkage was replaced by two carbamate moieties separated each other by a *N*,*N*′-dimethylethylenediamine linker (compound **2**, Fig. 3b). This modification significantly improved the overall stability of the probe but has a detrimental effect on the kinetics for fluorophore release. These observations led us to study the kinetic behavior of the PGA-catalysed disassembly process of several amidase-sensitive fluorogenic probes bearing various self-immolative spacers including PABA or hemithioaminal derivatives, in order to find the best compromise between stability, self-reactivity upon enzyme triggering, and substrate scopes and limitations. Here, we present the results of this comparative study whose ultimate goal is to select an optimised linker for the construction of protease-sensitive fluorogenic probes fulfilling all requirements for *in cellulo* or *in vivo* imaging applications.

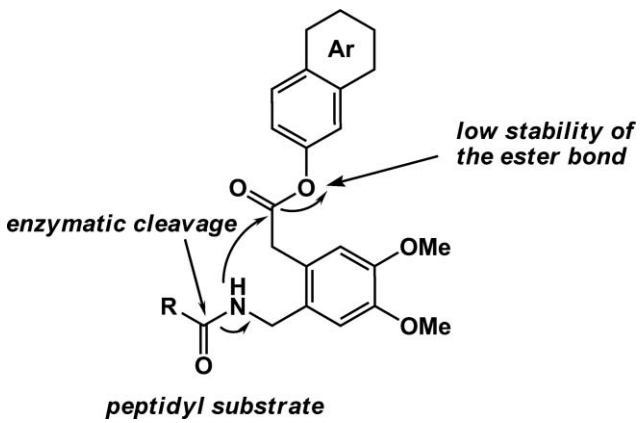


Fig. 2 General structure of phenol-based fluorogenic probes derived from the Waldmann traceless linker.

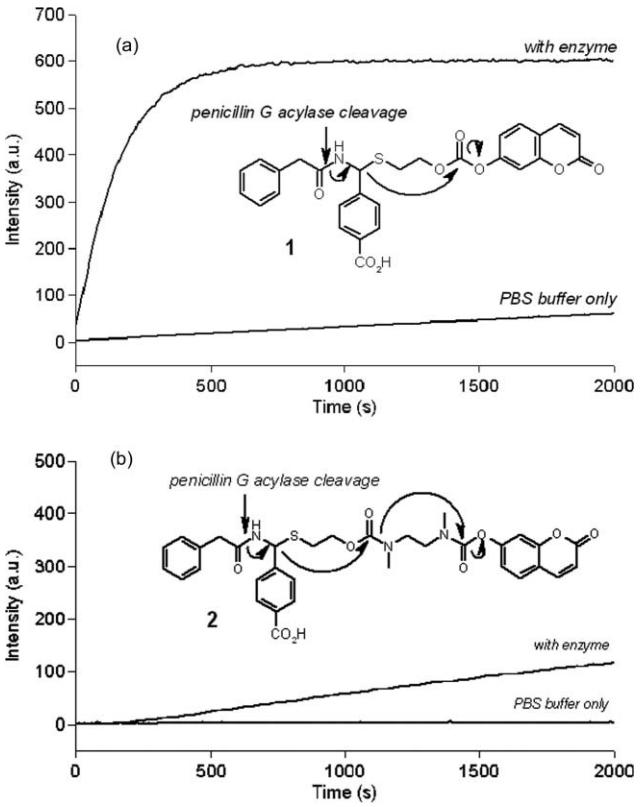
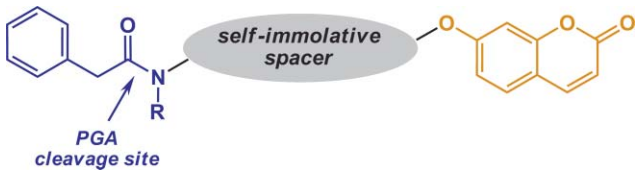


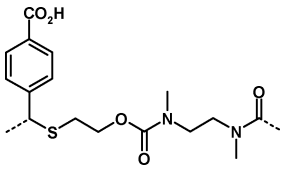
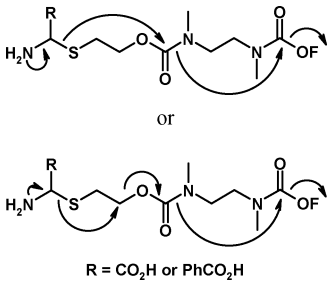
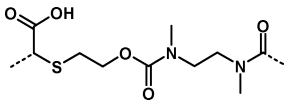
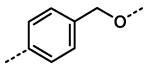
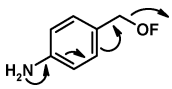
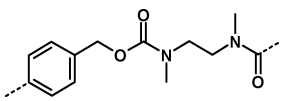
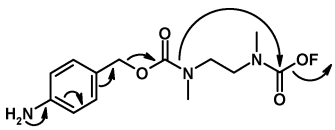
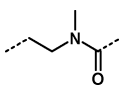
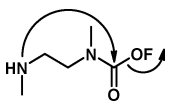
Fig. 3 a) Fluorescence emission time-course of pro-fluorescent compound **1** (concentration: 4.5 μM) with recombinant PGA (0.12 U, 37 °C) in PBS buffer at 460 nm ($\lambda_{ex} = 360$ nm). b) Fluorescence emission time-course of pro-fluorescent compound **2** (concentration: 3.0 μM) with recombinant PGA (0.12 U, 37 °C) in PBS buffer at 460 nm ($\lambda_{ex} = 360$ nm).

In this context, five model compounds **2**–**6** (Table 1) were synthesised (see ref. 14 for **2** and ref. 5 for **4**, see ESI† for synthesis details of **3**, **5** and **6**). These molecules were designed specifically to integrate: (1) a phenylacetamide moiety to be cleaved by PGA functioning as the triggering agent, and (2) a masked umbelliferone (*i.e.*, 7-hydroxycoumarin) unit to be released as the final product and to elicit turn-on fluorescence. Concerning the hemithioaminal derivative **3**, the starting material *para*-carboxybenzaldehyde used for the preparation of **1** and **2**, was replaced by glyoxilic acid in order to get an amino acid-like spacer (*i.e.*, α-substituted glycine) which may be further inserted within peptide architectures according to standard peptide synthesis protocols.[16] In addition to these PABA and hemithioaminal-based linkers which were elongated with the *N*,*N*′-dimethylethylenediamine moiety for compounds **2**, **3** and **5**, this latter bis-amine cyclisation spacer alone was equipped with phenylacetamide trigger and 7-hydroxycoumarin as reporter group to give **6**. Indeed, to our knowledge, there is no information in the literature about the ability of PGA to cleave such *N*-disubstituted peptide bond, and such a fluorogenic probe enables us to address this issue.

All these fluorogenic probes were incubated at the same concentration at 37 °C with PGA (0.12 U) in phosphate buffered saline (PBS, pH 7.5) and the corresponding fluorescence time-courses were recorded at $\lambda = 460$ nm (Fig. 4). Comparison of the resulting curves clearly indicates that the decomposition of PABA-based

Table 1 Disassembly mechanisms of self-immolative spacers PABA and hemithioaminal derivatives for the PGA-induced release of 7-hydroxycoumarin



Compound[a]	Self-immolative spacer	Reaction type	Release mechanism[b]
2		Fragmentation + dual cyclisation	
3			
4		1,6-Benzyl elimination	
5		1,6-Benzyl elimination + Cyclisation	
6		Cyclisation	

[a] R = H for compounds **2–5** and Me for compound **6** [b] OF = 7-hydroxycoumarin

self-immolative linkers was faster than with the hemithioaminal counterparts. Thus, the 1,6-benzyl elimination clearly occurs much more rapidly than the fragmentation-cyclisation process involving a thioalkyl carbamate intermediate. Yet, it is interesting to note that the elongated pro-fluorescent probe **5** was able to release the phenol-based fluorophore even faster than the parent PABA-based fluorogenic probe **4**. As already observed by Lee *et al.*,[12] the carbamate decarboxylation occurring during the domino reaction provides an additional thermodynamic driving force that is responsible for the experimentally observed faster fluorescence response of **5** compared to **4**. Similarly, pro-fluorescent probe **2** underwent a slower fragmentation when compared with compound **5** because the first cyclisation reaction involves a nucleophilic attack of the free thiol onto a less electrophilic carbamate which leads to the slow release of ethylene monothiocarbonate instead of CO_2 (see Table 1 for the different PGA-initiated domino reactions explored in this study). An alternative disassembly mechanism involving the internal nucleophilic attack of the thiol group on the α carbon of the 2-thioethyl moiety and subsequent elimination of ethylene episulfide and CO_2, can be also considered.[17] However, we and others have clearly demonstrated that the decomposition of 2-thioethyl carbamate intermediates (through one of these two disassembly mechanisms) is favoured by the formation of a thiolate anion, a better nucleophile than the corresponding thiol, and so readily occurred at pH over 8.0 and not under physiological conditions.[18] Pro-fluorescent probes **2** and **3** exhibit similar kinetics for the self-immolation process, revealing that some structural variations close to the active site of the enzyme did not interfere with the substrate recognition. However, enzymatic cleavage did not occur with fluorogenic probe **6**, suggesting that the *N*-methylation of the cleavable phenylacetamide bond prevents its hydrolysis by PGA. Furthermore, no nonspecific cleavage of these pro-fluorescent probes was detected in control reactions where **2–6** were incubated only in PBS (see ESI† for the corresponding fluorescence emission time-course curves). These results clearly demonstrate that the use of elongated PABA or hemithioaminal based linkers enables to get enzyme-reactive pro-fluorophores with high chemical stability.

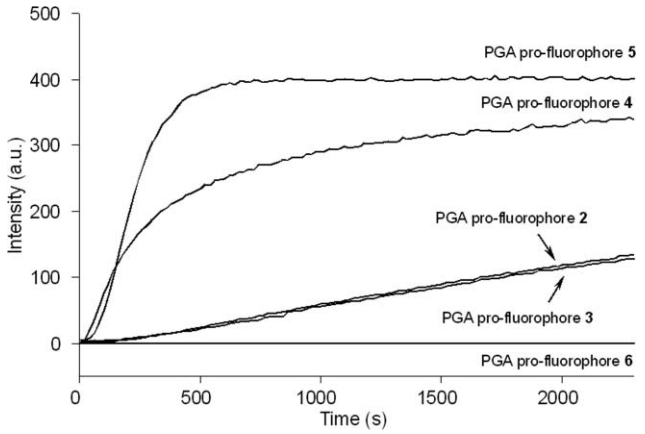


Fig. 4 Fluorescence emission time-course of pro-fluorescent probes **2–6** (concentration: 3.0 μM) with recombinant PGA (0.12 U, 37 °C) in PBS buffer at 460 nm (λ_{ex} = 360 nm).

In conclusion, self-immolative linkers based on the hemithioaminal core disassemble slower than PABA analogues when using *N*,*N*′-dimethylethylendiamine as a stabilising spacer. Thus, PABA derivatives will be used for getting fast-response probes whereas the hemithioaminal spacers will be preferred for the construction of tunable (bio)functionalised probes. Indeed, these latter compounds exhibit a conveniently positioned carboxylic acid group which may be used as an anchoring point for grafting either: (1) a water-solubilising group such as polysulfonated peptide-based linkers[19] especially to counterbalance the hydrophobicity of the grafted fluorophore, (2) an additional peptide as part of the substrate for endoproteases, to increase the enzyme specificity/selectivity or binding or (3) a targeting ligand suitable for driving the designed pro-fluorescent probe or prodrug to the targeted tissue and/or proteolytic enzyme. The present study thus both widens the scope of accessible self-immolative linkers, and provides additional informations to those recently published by the groups of Lee, Shabat and Warnecke. This thus should aid the choice and design of suitable self-immolative systems useful for the development of optical bioprobes or drug delivery vehicles suitable, for instance, for cancer imaging and chemotherapy.

Acknowledgements

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