ORGANIC LETTERS

2008
Vol. 10, No. 8
1517−1520

Development of a New Nonpeptidic Self-Immolative Spacer. Application to the Design of Protease Sensing Fluorogenic Probes

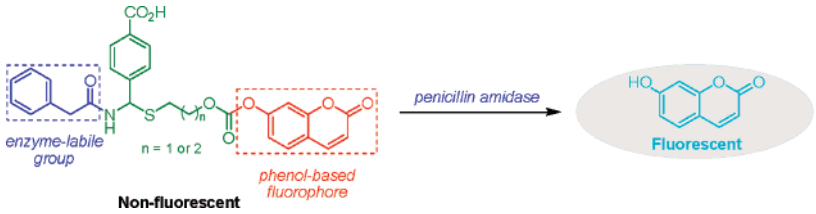
Yves Meyer,[†,‡] Jean-Alexandre Richard,[†,‡] Marc Massonneau,[‡] Pierre-Yves Renard,*[,†,§] and Anthony Romieu*[,†,§]

Equipe de Chimie Bio-Organique, Université de Rouen, Place Emile Blondel, 76821 Mont-Saint-Aignan, France, IRCOF, UMR 6014, rue Lucien Tesnière, 76131 Mont-Saint-Aignan, France, and QUIDD, Technopôle du Madrillet, 50, rue Ettore Bugatti, 76800 Saint-Etienne du Rouvray, France

pierre-yves.renard@univ-rouen.fr

Received January 28, 2008

ABSTRACT



The design and synthesis of novel self-immolative spacer systems aiming at the release of phenol-containing compounds are described. The newly designed traceless linkers proved to be conveniently stable under physiological conditions and operate through spontaneous decomposition of an hemithioaminal intermediate under neutral aqueous conditions. Their utility was then illustrated by the preparation of original fluorogenic substrates of penicillin amidase whose strong fluorescence is unveiled through enzyme-initiated domino reactions.

During the past decade, traceless linkers have emerged as powerful tools in solid-phase organic synthesis, especially for the rapid production of highly diverse organic compound libraries created through combinatorial or parallel chemistry methods.[1] Traceless linkers are so-called because their cleavage results in an aliphatic or aromatic hydrogen atom, so that an examination of the final compound reveals no trace of the point of linkage to the solid phase (unlike classical cleavable linkers that generally leave functionalities such as carboxylic acid, carboxamide, ...). Recent innovations in the chemistry of such linkers and especially the use of enzymes to release the desired products under very mild neutral and aqueous conditions,[2] have enabled the broadening of the scope of these chemical tools to the preparation of efficient drug delivery agents for pharmaceuticals applications,[3] as well as various reversible bioconjugates and sophisticated spectroscopic bioprobes for applications in the field of biological analysis.[4,5] Regarding these latter multicomponents (bio)molecular systems, traceless linkers (the term "self-immolative spacer" is also used in this context) are used as reversible covalent bonds between two molecular species: as their self-decomposition, often triggered by enzyme-initiated domino reactions,[6] leaves no further reactive

[†] COBRA−CNRS UMR 6014.
[‡] QUIDD (QUantitative Imaging in Drug Development).
[§] Université de Rouen.

(1) Gil, C.; Bräse, S. *Curr. Opin. Chem. Biol.* **2004**, *8*, 230−237. Wills, A. J.; Balasubramanian, S. *Curr. Opin. Chem. Biol.* **2003**, *7*, 346−352. Comely, A. C.; Gibson, S. E. *Angew. Chem., Int. Ed.* **2001**, *40*, 1012−1032. Bräse, S.; Dahmen, S. *Chem. Eur. J.* **2000**, *6*, 1899−1905.
(2) Reents, R.; Jeyaraj, D. A.; Waldmann, H. *DDT* **2002**, *7*, 71−76.
(3) Kratz, F.; Müller, I. A.; Ryppa, C.; Warnecke, A. *ChemMedChem* **2008**, *3*, 20−53.
(4) Chen, X.; Sun, M.; Ma, H. *Curr. Org. Chem.* **2006**, *10*, 477−489.
(5) Goddard, J.-P.; Reymond, J.-L. *Trends Biotechnol.* **2004**, *22*, 363−370.
(6) Mayer, S. F.; Kroutil, W.; Faber, K. *Chem. Soc. Rev.* **2001**, *30*, 332−339.

10.1021/ol800198f CCC: $40.75
Published on Web 03/22/2008

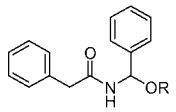
functionality, the structure and properties of each released molecule are indeed guaranteed to be the same as before conjugation.

In the area of fluorescent bioprobes, recent work has clearly shown that self-immolative spacers are key components in the smart design and synthesis of latent fluorophores, exhibiting valuable properties for the illumination of numerous biochemical processes.[7] Indeed, these three-component probes (trigger-linker-fluorophore) unmask their intense fluorescence only by a specific enzymatic cleavage that produces a labile self-immolative linker-fluorophore derivative that in turn eliminates spontaneously to release the original fluorescent marker. These latent fluorophores thus display a unique selectivity and limited interferences associated with the probe concentration, excitation intensity, and emission sensitivity.

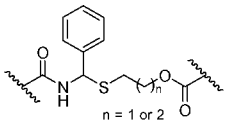
Some latent fluorophores of proteolytic enzymes have been reported[4] based on the direct linkage between a fluorophore moiety and an enzyme recognition unit but the absence of a spacer between the two compartments happened to cause two main problems: (1) pro-fluorophores designed for enzymatic detection have often revealed to be poor substrates for their respective enzymes; these problems are caused by steric hindrance brought by the presence of the (bulky) fluorophore molecule next to the cleavage site which may prevent access to the active pocket of the enzyme; (2) the choice of fluorophores is almost exclusively restricted to aromatic amines such as 7-amino-4-methylcoumarin, cresyl violet, and rhodamine 110, which do not cover a wide range of detection wavelengths in the visible and near-infrared spectra. To try and solve the aforementioned limitation (1), Jones et al.[8] successfully described the use of a reactive linker for the design of an image contrast agent selectively activated by the serine protease PSA (i.e., prostate specific antigen), but the approach turned out to be only applicable to amine-based fluorophores.

Thus, there is a growing need for original self-immolative spacers that enable the reversible conjugation of a peptide substrate to a fluorophore bearing either a reactive amino, hydroxyl, or thiol group. In that context, we want to describe herein our efforts to design and evaluate a new self-immolative linker.

The design of the linker was based on the improvement of the enzyme-labile linker **1** initially developed by Böhm et al. for the reversible covalent attachment of alcohols to solid supports.[9] As the chemical derivatization of the activated form of this linker (i.e., benzotriazole derivative) with aniline, phenol, or thiophenol derivatives is tricky and leads to unstable conjugates, we chose to introduce stable and non activated thioalkyl moieties onto this masked hemiaminal, yielding the corresponding *N*-acylhemithioaminal **2**. In order to have a self-immolative linker, the chosen thioalkyl moiety was obtained by introducing a thioalkyloxycarbonyl (namely, 2-thioethyl- or 3-thiopropyloxycarbonyl). These latter linkers have been recently used by us for the design of thiol-labile amine and alcohol protecting groups[10] and by other academic-industry research teams for the preparation of bioconjugates suitable for bioimaging[11] and drug delivery applications.[12] The mechanism leading to their self-decomposition has been intensively studied and protocols allowing their conjugation with many *N*- and *O*-nucleophiles involving the corresponding activated carbonate derivatives are now well established.



Enzyme-labile linker **1**
(Böhm *et al.* **1998**)



Self-immolative *N*-acylhemithioaminal **2**

We report here the synthesis and evaluation of three such water-soluble self-immolative spacers derived from the original *N*-acylhemithioaminal core structure **2** and their application for the preparation of latent fluorescent probes aimed at the detection of a model protease, penicillin amidase (also known as penicillin G acylase, PGA).

In the design of the traceless linker, the biocatalyzed transformation (i.e., the enzymatic hydrolysis of a carboxamide bond) is thus combined with a domino reaction involving fragmentation of the hemithioaminal **5** and subsequent self-cyclization of the resulting thioalkyl carbonate **6**, yielding to the release of the phenol derivatives (Figure 1). To validate the postulated mechanism of this cascade reaction and to check its efficacy under physiological conditions, we have chosen to work with PGA, a commercially available and widely used biocatalyst in the enzymatic synthesis of β-lactam antibiotics, since it allows for the deprotection of phenacetyl-protected amines[13] and the subsequent release of umbelliferone (i.e., 7-hydroxycoumarin),[5] at the blue fluorescent phenolic fluorophore. Thus, it was possible to follow the enzymatic hydrolysis and the following decomposition of the self-immolative spacer by means of a simple and rapid in vitro fluorescence assay. In addition to these structural features, the low water solubility of first generation probes drove us to design fluorogenic probes **3** and **4**, bearing an additional carboxyl group on the aromatic part of the masked benzyl hemithioaminal.

(7) Ho, N.-H.; Weissleder, R.; Tung, C.-H. *ChemBioChem* **2007**, *8*, 560−566. Lavis, L. D.; Chao, T.-Y.; Raines, R. T. *ChemBioChem* **2006**, *7*, 1151−1154. Lavis, L. D.; Chao, T.-Y.; Raines, R. T. *ACS Chem. Biol.* **2006**, *1*, 252−260. Chandran, S. S.; Dickson, K. A.; Raines, R. T. *J. Am. Chem. Soc.* **2005**, *127*, 1652−1653.

(8) Jones, G. B.; Crasto, C. F.; Mathews, J. E.; Xie, L.; Mitchell, M. O.; El-Shafey, A.; D'Amico, A. V.; Bubley, G. J. *Bioorg. Med. Chem.* **2006**, *14*, 418−425.

(9) Böhm, G.; Dowden, J.; Rice, D. C.; Burgess, I.; Pilard, J.-F.; Guilbert, B.; Haxton, A.; Hunter, R. C.; Turner, N. J.; Flitsch, S. L. *Tetrahedron Lett.* **1998**, *39*, 3819−3822.

(10) Lapeyre, M.; Leprince, J.; Massonneau, M.; Oulyadi, H.; Renard, P.-Y.; Romieu, A.; Turcatti, G.; Vaudry, H. *Chem. Eur. J.* **2006**, *12*, 3655−3671.

(11) Jones, L. R.; Goun, E. A.; Shinde, R.; Rothbard, J. B.; Contag, C. H.; Wender, P. A. *J. Am. Chem. Soc.* **2006**, *128*, 6526−6527.

(12) El Alaoui, A.; Schmidt, F.; Amessou, M.; Sarr, M.; Decaudin, D.; Florent, J.-C.; Johannes, L. *Angew. Chem., Int. Ed.* **2007**, *46*, 6469−6472. Vlahov, I. R.; Santhapuram, H. K. R.; Kleindl, P. J.; Howard, S. J.; Stanford, K. M.; Leamon, C. P. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5093−5096. Henne, W. A.; Doorneweerd, D. D.; Hilgenbrink, A. R.; Kularatne, S. A.; Low, P. S. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5350−5355.

(13) Ninkovic, M.; Riester, D.; Wirsching, F.; Dietrich, R.; Schwienhorst, A. *Anal. Biochem.* **2001**, *292*, 228−233.

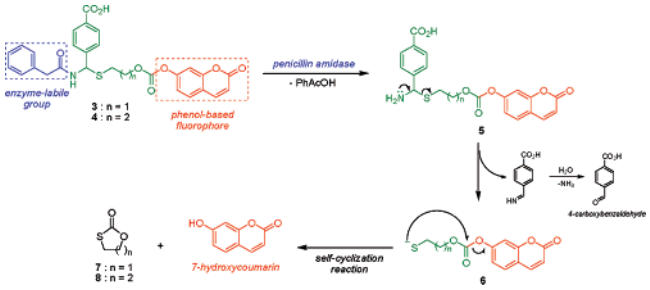
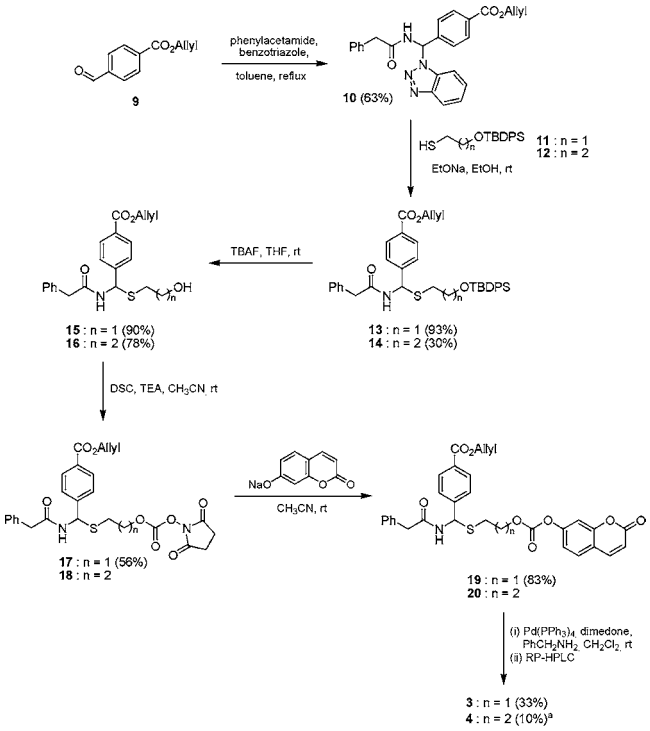


Figure 1. Principle of the original self-immolative linker studied in this work and its application to the release of phenol-based fluorophores after carboxamide bond cleavage.

The synthesis of self-immolative spacers and their chemical derivatization with the fluorescent reporter group was achieved as depicted in Scheme 1. First, the enzyme

Scheme 1. Synthesis of Fluorogenic Probes **3** and **4**[a]



[a] Overall yield for the three last steps because the synthesis of fluorogenic probe **4** was performed in a small scale without isolation of intermediates **18** and **20** in a pure form.

recognition unit (i.e., phenylacetamide moiety) was introduced onto the activated form of the linker by using a methodology developed by Katritzky et al.[14] The three-component reaction was performed with the allyl ester of 4-carboxybenzaldehyde **9**: the allyl protecting group was selected since it was proven to be stable to the acidic and basic conditions encountered during the synthesis of **3** and **4** and could be selectively removed by treatment with Pd-$(PPh_3)_4$ and dimedone without affecting the acid-sensitive *N*-acylhemithioaminal moiety and the fragile carbonate derived from phenol-containing fluorophores. The benzotriazole derivative **10** was prepared in 63% yield by refluxing a mixture of aldehyde **9**, benzotriazole, and phenylacetamide in dry toluene for 3 days. Reaction of **10** with the sodium salt of *O*-(*tert*-butyldiphenylsilyl)-protected derivative of β-mercaptoethanol **11** or 3-sulfanylpropanol **12** in dry ethanol afforded the full-protected hemithioaminals **13** and **14** in 93% and 30% yields, respectively.[15] The modest yield obtained in the case of the preparation of compound **14** was explained by a competitive transesterification of the allyl ester. Removal of the *tert*-butyldiphenylsilyl (TBDPS) group was achieved under standard conditions by treatment of **13** and **14** with a 2-fold excess of tetrabutylammonium fluoride (TBAF) in THF to give the corresponding alcohols **15** and **16**. Activation of alcohols **15** and **16** with *N*,*N*′-disuccinimidyl carbonate (DSC) in dry acetonitrile in the presence of triethylamine afforded carbonates **17** and **18**. Their reaction with the sodium salt of umbelliferone gave the non water-soluble coumarin-based fluorogenic probes **19** and **20** in good yields. Finally, the allyl esters of **19** and **20** were removed under palladium catalysis in the conditions reported by Zhang et al.[16] The use of a weak nucleophile (i.e., a 3:2 (mol/mol) mixture of dimedone/benzylamine) as the allyl scavenger was required to minimize the undesirable nucleophile-mediated cleavage of the sensitive carbonate moiety. Purification was achieved by semipreparative RP-HPLC to give the resulting water-soluble PGA sensitive probes **3** and **4** in moderate yields (33% for **3** and 10% overall yield for the last three steps for **4**). All spectroscopic data (see the Supporting Information), especially NMR and mass spectrometry, were in agreement with the structures assigned.

Fluorogenic probes **3** and **4** were evaluated under simulated physiological conditions (PBS, pH 7.5, see Figure 2 and the Supporting Information). As expected, profluorescent probe **3** exhibited no significant fluorescence emission at 458 nm (diminished by 30-fold compared to the fluorescence of free umbelliferone at the same concentration, see Figure 2A). Figure 2B showed the time-course for the enzyme catalyzed hydrolysis of **3**. After adding recombinant PGA to the substrate solution, a strong fluorescence signal generated at 460 nm indicated the catalytic cleavage of the carboxamide bond and the release of free umbelliferone. The maximum fluorescence intensity reflecting the quantitative conversion of **3** into 7-hydroxycoumarine was reached after about 10 min. When substrate **3** was incubated in PBS only, a slow linear increase in fluorescence intensity was observed, corresponding to the nonenzymatic hydrolysis of its carbonate moiety. The moderate stability of monoaryl carbonates derived from β-mercaptoethanol in aqueous buffers has already been observed by Jones et al. in the context of

(14) Katritzky, A. R.; Drewniak, M.; Lue, P. *J. Org. Chem.* **1988**, *53*, 5854−5856.

(15) Katritzky, A. R.; Takahashi, I.; Fan, W. Q.; Pernak, J. *Synthesis* **1991**, 1147−1150.

(16) Zhang, H. X.; Guibé, F.; Balavoine, G. *Tetrahedron Lett.* **1988**, *29*, 623−626.

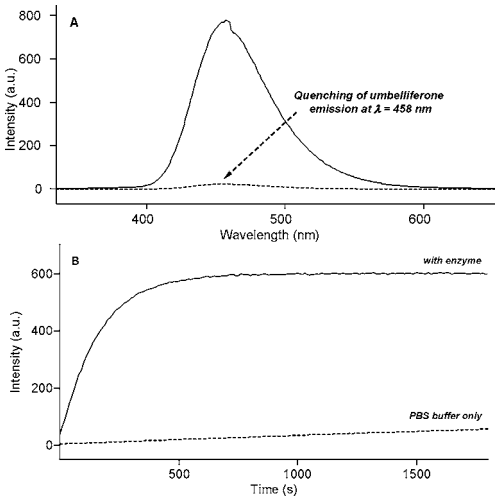
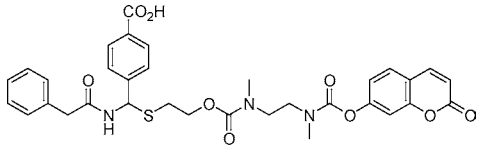


Figure 2. (A) Emission spectra of (ex λ = 323 nm) of umbelliferone (−) and fluorogenic probe **3** (- - -) at 25 °C in PBS (concentration 2.4 μM). (B) Fluorescence emission time-course of probe **3** (concentration 3.0 μM) with recombinant PGA (0.12 U, incubation time 30 min, 37 °C) in PBS buffer at 460 nm (ex λ = 360 nm).

releasable luciferin-transporter conjugates[11] and could be partially circumvented by the lengthening of the alkyl chain between the sulfur atom and the carbonate group. Indeed, when the fluorogenic probe **4** derived from 3-sulfanylpropanol was incubated in PBS, a slow linear increase in fluorescence intensity at 460 nm was also observed but the nonspecific hydrolysis rate decreased significantly compared to **3** (ratio between slopes **4**/**3**: 0.7), whereas the specific enzymatic cleavage showed equivalent kinetics (see the Supporting Information).

In addition to these spectral measurements, LC−MS analyses of the enzymatic hydrolysis mixture of **3** were performed to confirm the enzyme-initiated self-decomposition mechanism of the *N*-acylhemithioaminal linker and proved both the identity of the released compounds 4-carboxybenzaldehyde (t_R = 6.4 min, $[M - H]^-$ = 149.11) and 7-hydroxycoumarin (t_R = 14.3 min, $[M - H]^-$ = 161.09) and the efficacy of this release process (see the Supporting Information).

To further improve the stability of enzyme-activable conjugates bearing the self-immolative *N*-acylhemithioaminal **2** toward nonspecific hydrolysis, we also explored the synthesis of an elongated PGA fluorogenic probe **21** that contained a well-known bis-amine cyclization spacer (see the Supporting Information). Indeed, carbamate linkages are known to be more stable than the corresponding carbonate moieties and the *N*-methyl-*N*-[2-(methylamino)ethyl] carbamate linker is one of the most popular self-immolative spacer for the release of alcohols and phenols especially in the context of prodrugs.[17] In vitro fluorescence assay and LC−MS analyses (see the Supporting Information) showed that **21** is not prone to nonspecific hydrolysis and was suitable for PGA sensing, although the kinetics of the enzyme triggered domino reaction was significantly slowed down.



Elongated PGA fluorogenic probe **21**

In summary, we have designed and synthesized a novel self-immolative spacer system that allowed the release of phenol-containing compounds through an original two-step cascade reaction triggered by the enzymatic formation of a free amine. The potential utility of this traceless linker strategy in bioconjugate chemistry was illustrated by the preparation of three fluorogenic probes aimed at the detection of the activity of a model enzyme penicillin amidase. Interestingly, the water-solubilizing carboxyl group of these acylhemithioaminal derivatives could be alternatively used for anchoring a targeting ligand suitable for guiding the profluorophore or the prodrug to the tumor-expressed proteolytic enzyme, aimed at designing optical bioprobes or drug delivery systems suitable for cancer imaging and chemotherapy.[18]

Acknowledgment. This work was supported by La Région Haute-Normandie and QUIDD. We thank Christophe Portal (QUIDD) and Freddy Rivault (QUIDD) for careful reading and English corrections of the manuscript, POLYINTELL company for the open access to their LC−MS apparatus and Dr. Céline Pérollier (Project Leader, POLYINTELL) for her technical assistance during LC−MS analyses.

Supporting Information Available: Procedures and additional data for syntheses and analyses reported herein. This material is available free of charge via the Internet at http://pubs.acs.org.

OL800198F

(17) Saari, W. S.; Schwering, J. E.; Lyle, P. A.; Smith, S. J.; Engelhardt, E. L. *J. Med. Chem.* **1990**, *33*, 97−101.

(18) For an example of such drug delivery system, see: Gopin, A.; Pessah, N.; Shamis, M.; Rader, C.; Shabat, D. *Angew. Chem., Int. Ed.* **2003**, *42*, 327−332.