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Chemiluminescent Probe for the in Vitro Detection of Protease Activity

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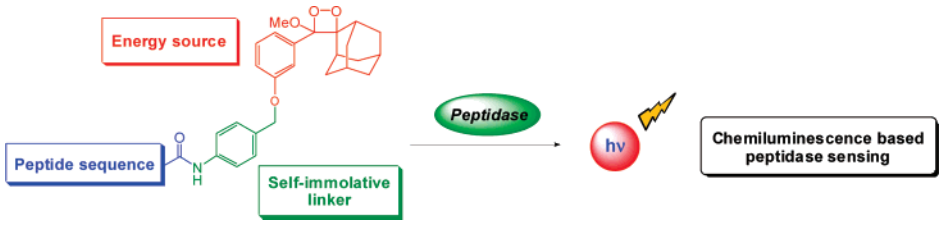
ABSTRACT



A strategy involving the use of a self-immolative linker has been investigated for the chemiluminescent sensing of proteases. The reactive linker enabled the release of a 1,2-dioxetane light precursor. As a proof of principle, caspase-3, a key peptidase involved in apoptosis has been targeted. An in vitro assay has been carried out and proved the decomposition of the linker and the selectivity for caspase-3.

Chemiluminescence is defined as the electromagnetic radiation (ultraviolet, visible, or infrared) produced when a chemical reaction yields an electronically excited intermediate or product, which either luminesces (direct chemiluminescence) or donates its energy to another molecule responsible for the emission (indirect or sensitized chemiluminescence). Oxalate esters, luminol, acridinium esters, and 1,2-dioxetanes are well-known chemiluminescent species emitting light following a chemical reaction.[1] The advantage of using chemiluminescence in bioanalytical assays is associated with a rapid and sensitive detection of the target through reduction of the background noise thanks to the absence of a photonic excitation. Thus, chemiluminescent probes, especially those derived from 1,2-dioxetanes **1**, have been designed for the highly sensitive detection of various enzymes such as alkaline phosphatase, β-galactosidase, neuramidinase.[2] Taking into account the versatility, the sensitivity, and the higher specific localization and quantification of target analytes in tissue selections offered by chemiluminescence as compared to those by fluorescence, chemiluminescent probes have been also used for the development of immuno- and immunohistoassays.[3] In order to widen the scope of chemiluminescent probes, our interest is focused on the development of chemiluminescent assays for new classes of enzymes. We have thus previously reported the design and synthesis of a chemiluminescent probe for the detection of acetylcholinesterase activity using an unprecedented thiophenol trigger.[4] We report here our first investigations regarding the tricky detection of proteases.

Despite recent advances in the development of chemiluminescent probes,[5] the detection of proteases still remains challenging. The issue mainly lies in the difficulty to obtain an efficient light signal following the cleavage of the peptide

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(1) Beck, S.; Koster, H. *Anal. Chem.* **1990**, *62*, 2258−2270; *Erratum* **1991**, *63*, 848.
(2) Matsumoto, M. *J. Photochem. Photobiol., C* **2004**, *5*, 27−53 and references therein.
(3) Kricka, L. J. *Anal. Chim. Acta* **2003**, *500*, 279−286.
(4) Sabelle, S.; Renard, P.-Y.; Pecorella, K.; de Suzzoni-Dezard, S.; Creminon, C.; Grassi, J.; Mioskowski, C. *J. Am. Chem. Soc.* **2002**, *124*, 4874−4880.
(5) For a recent example, see Hewage, H. S.; Wallace, K. J.; Anslyn, E. V. *Chem. Commun.* **2007**, 3909−3911.

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bond. 1,2-Dioxetanes indeed usually emit light through spontaneous decomposition of a compound such as **2**, bearing a negatively charged atom onto an aromatic ring bearing the 1,2-dioxetane, via a mechanism chemically initiated electron exchange luminescence (CIEEL). **2** can be, in the case of enzymes probes, formed specifically through the enzymatic cleavage of **1** (Figure 1). One possible approach for the

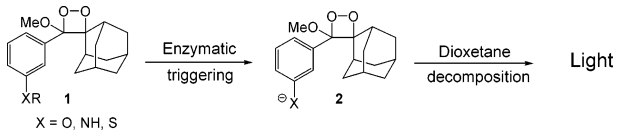


Figure 1. Enzymatic activation of **1** leading to **2** whose decomposition triggers light emission.

detection of proteases would thus be to connect the C-terminus of a peptide sequence directly to the aromatic moiety (i.e., X = NH). However, the amount of light emitted by the release of aniline is by far much weaker than that by the phenol or thiophenol equivalent (i.e., X = O or S).[6]

Thus, inspired by the chemistry of prodrugs widely used in medicinal chemistry for drug delivery applications,[7] we designed probe **3** which incorporates a reactive linker able to transfer the amide bond-breaking event to a phenolate release (Figure 2). *p*-Aminobenzyl alcohol **8** (PABA) was

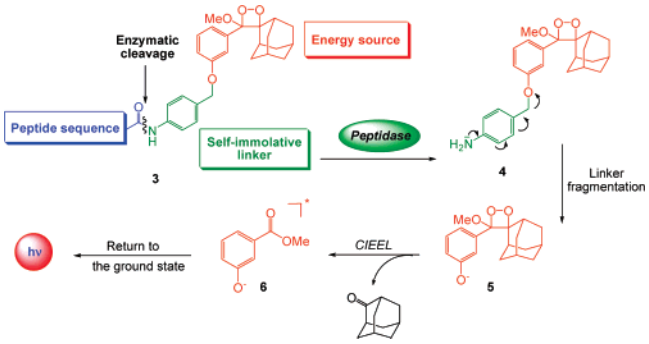


Figure 2. Self-immolative linker strategy for the release of the phenolate moiety **5** after peptide bond cleavage.

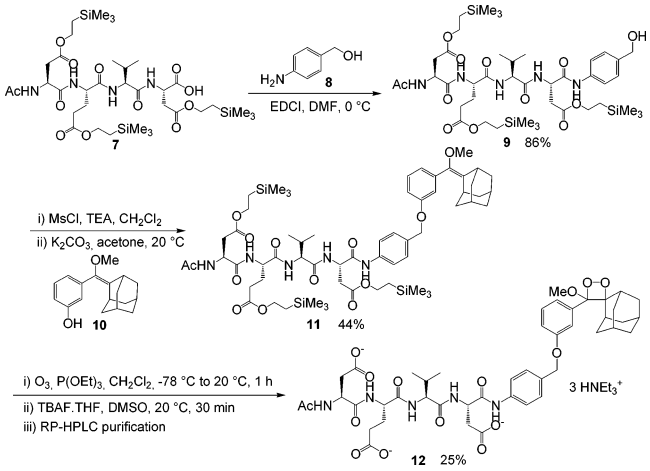
chosen as a self-immolative linker.[8] Thus, aniline **4**, formed by the amide bond cleavage, should spontaneously decompose to release phenolate **5**, leading to the excited compound **6**, which returns to the ground state through light emission.

As a proof of concept, we applied our device to the sensing of caspase-3, an effector peptidase which plays a crucial role in the last steps of the apoptotic process.[9] Although many fluorescent caspase-3-activity probes have been reported so far,[10] none of them is based on chemiluminescence. Moreover, other caspases have a close substrate profile as compared to casapse-3; thus, providing that the probe shows a high sequence selectivity, this strategy should allow the construction of a flexible set of caspase chemiluminescent substrates. This enzyme thus appeared to be an interesting case study before extending our device to other proteases.

Hence, the linker **8** was introduced onto the DEVD (for Asp-Glu-Val-Asp) peptide sequence **7** which is specifically recognized by caspase-3 and cleaved after the Asp residue at the C-terminal side.[11] **7** was prepared in a highly efficient 13-step procedure by solution peptide synthesis using a Boc strategy (93% mean yield for each step, see Supporting Information [SI]). In order to have an orthogonal set of protective groups, the three carboxylic acids of peptide **7** were protected as 2-trimethylsilylethyl (TMSE) esters,[12] easily removed by treatment with fluoride ion sources under mild conditions, shown to be compatible with the 1,2-dioxetane structures.

Then, benzylic alcohol **9** was converted to its mesylate derivative, and enol ether **10**, prepared from 3-hydroxybenzoic acid methyl ester in three steps according to literature procedures,[13] was incorporated on the C-terminus of peptide−benzyl alcohol **9** (Scheme 1).

Scheme 1. Synthesis of Chemiluminescent Probe **12**



Finally, compound **11** underwent the key step of the synthesis: a [2+2] cycloaddition between enol ether **11** and singlet oxygen ($^1O_2$).[4] The formation of the 1,2-dioxetane derivative was checked by RP-HPLC, and the compound was subsequently isolated in a pure form by semipreparative RP-HPLC. Final deprotection with tetrabutylammonium fluoride

(6) (a) Bronstein, I.; Edwards, B.; Martin, C.; Sparks, A.; Voyta, J. C. (Tropix, Inc., U.S.A.). WO 9624849, 1996. (b) Watanabe, N.; Ichikawa, M.; Ono, A.; Murakami, H.; Matsumoto, M. *Chem. Lett.* **2005**, *34*, 718−719.

(7) (a) Damen, E. W. P.; Nevalainen, T. J.; van den Bergh, T. J. M.; de Groot, F. M. H.; Scheeren, H. W. *Bioorg. Med. Chem.* **2001**, *10*, 71−77. (b) de Groot, F. M. H.; Loos, W. J.; Koekkoek, R.; van Berkom, L. W. A.; Busscher, G. F.; Seelen, A. E.; Albrecht, C.; de Bruijn, P.; Scheeren, H. W. *J. Org. Chem.* **2001**, *66*, 8815−8830. (c) Amir, R. J.; Pessah, N.; Shamis, M.; Shabat, D. *Angew. Chem., Int. Ed.* **2003**, *42*, 4494−4499. (d) Haba, K.; Popkov, M.; Shamis, M.; Lerner, R. A.; Barbas, C. F., III; Shabat, D. *Angew. Chem., Int. Ed.* **2005**, *44*, 716−720. (e) Amir, R. J.; Danieli, E.; Shabat, D. *Chem. Eur. J.* **2007**, *13*, 812−821. (f) Shamis, M.; Shabat, D. *Chem. Eur. J.* **2007**, *13*, 4523−4528.

(TBAF) led to chemiluminescent probe **12** after purification by semipreparative RP-HPLC. The structure of this chemiluminescent caspase-3 substrate was confirmed by ESI mass spectrometry (see SI).

With this tool in hand, we investigated the efficiency of our device against recombinant human caspase-3. As the chemiluminescence of a 1,2-dioxetane happens to be less efficient in an aqueous media compared to organic solvents,[14] specific enhancers (namely 5-(stearoylamino)fluorescein (emission wavelength = 530 nm) as well as a surfactant cetyltrimethylammonium bromide [CTAB]) were added to the medium. Thus, caspase-3-mediated cleavage of probe **12** was carried out following a classical final time detection procedure, and a significant light emission at 530 nm could be observed (see SI). The light was persistent for more than 10 min, corresponding to a "glow" chemiluminescence which is known to allow a more sensitive detection than a "flash" emission.[1] The long-lived time course of this light emission is thus an interesting feature for further biological assays (Figure 3A). The maximum of light emission was also represented as a function of time, thus enabling us to follow the kinetics of the enzymatic reaction (Figure 3B).

Finally, the caspase-3 detection limit in this nonoptimized assay format was estimated by decreasing the amount of enzyme until no more light could be significantly detected (see SI). Hence, an encouraging detection limit of 1.31 pmol of enzyme was determined (Figure 3C). Furthermore, control reactions in which probe **12** was incubated with caspase-3 buffer alone, with penicillin amidase (penicillin G acylase), or with other initiator caspases were undertaken. In these conditions, no emission of light was detected, showing that neither spontaneous hydrolysis nor nonspecific enzymatic cleavage of **12** occurred (see SI).

In conclusion, we have designed and synthesized an efficient chemiluminescent probe suitable for the in vitro detection of protease activity. A strategy involving the use of a self-immolative spacer **8** (PABA) was developed to release the light precursor phenolate **5**. An application of this device was undertaken, and the first chemiluminescent caspase-3 probe **12** was synthesized. An in vitro assay enabled the detection of a "glow" luminescence suitable for further biological applications. Moreover, preliminary assays proved that the probe was selective and exhibited a detection limit of around 1 pmol. This new tool constitutes a great innovation in the field of protease-sensing assays and optical bioprobes. Currently, work is in progress in our laboratory to extend this device to other proteases of biological interest.

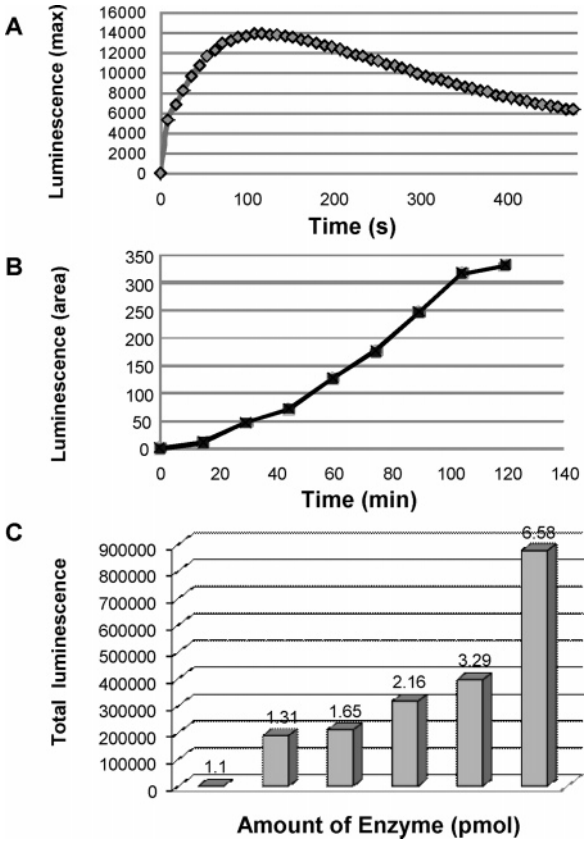


Figure 3. (A) Luminescence intensity (area under the emission curve in the range 490−570 nm) recorded every 9 s with probe **12** after incubation with recombinant human caspase-3 ($1.6\ 10^{-3}$ U, incubation time 105 min). (B) Maximum light emission of probe **12** as a function of the incubation time with recombinant human caspase-3. (C) Determination of the detection limit of caspase-3.

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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.

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(8) Carl, P. L.; Chakravarty, P. K.; Katzenellenbogen, J. A. *J. Med. Chem.* **1981**, *24*, 479−480.
(9) Denault, J.-B.; Salvesen, G. S. *Chem. Rev.* **2002**, *102*, 4489−4499.
(10) (a) Wang, Z.-Q.; Liao, J.; Diwu, Z. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2335−2338 and references cited therein. (b) Bouteiller, C.; Clave, G.; Bernardin, A.; Chipon, B.; Massonneau, M.; Renard, P.-Y.; Romieu, A. *Bioconjugate Chem.* **2007**, *18*, 1303−1317 and references 55−58 therein.
(11) (a) Philchenkov, A. A. *Biochemistry (Moscow)* **2003**, *68*, 365−376. (b) Fuentes-Prior, P.; Salvesen, G. S. *Biochem. J.* **2004**, *384*, 201−232.
(12) (a) Gerlach, H. *Helv. Chim. Acta* **1977**, *60*, 3039−3044. (b) Sieber, P. *Helv. Chim. Acta* **1977**, *60*, 2711−2716.
(13) (a) Sabelle, S.; Hydrio, J.; Leclerc, E.; Mioskowski, C.; Renard, P.-Y. *Tetrahedron Lett.* **2002**, *43*, 3645−3648. (b) Bastos, E. L.; Ciscato, L. F. M. L.; Weiss, D.; Beckert, R.; Baader, W. J. *Synthesis* **2006**, 1781−1786. (c) Renard, P.-Y.; Romieu, A.; Massonneau, M. (Quidd, Fr.; Universite De Rouen). WO 2886292, 2006.
(14) (a) Schaap, A. P.; Louis, H. A.; Romano, J. *Clin.Chem.* **1989**, 1863−1864. (b) Schaap, A. P. (Wayne State University, U.S.A.). EP 0352713, 1990. (c) Adam, W.; Bronstein, I.; Edwards, B.; Engel, T.; Reinhardt, D.; Schneider, F. W.; Trofimov, A. V.; Vasil'ev, R. F. *J. Am. Chem. Soc.* **1996**, *118*, 10400−10407.