Self-cleavable chemiluminescent probes suitable for protease sensing†

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A new generation of dioxetane-based chemiluminescent substrates suitable for detecting protease activities is described. Our strategy involves the use of a self-cleavable spacer as the key molecular component of these protease-sensitive chemiluminescent probes. Among the assayed strategies, the PABA (*para*-aminobenzylic alcohol) linker associated with an ether linkage enables the release of the light-emitting phenolic 1,2-dioxetane moiety through an enzyme-initiated domino reaction. To validate this strategy, two proteolytic enzymes were chosen: penicillin amidase and caspase-3, and the corresponding self-cleavable chemiluminescent substrates were synthesised. Their evaluation using an *in vitro* assay has enabled us to prove the decomposition of the linker under physiological conditions and the selectivity for the targeted enzyme.

Introduction

From the human genome data, it is now known that more than 500 genes encode proteases.[1] Proteases mediate non specific protein hydrolysis, but also have a vital role in post-translational modifications of proteins, performing selective and efficient cleavage of specific substrates.[2] In addition, the expression and activity of such proteolytic enzymes are significantly up-regulated in several pathologies, including cancer, arthritis and atherosclerosis. They can hence be considered as key biological markers for these pathologies. Synthetic probes for the detection of protease activity are therefore essential, and most probes use fluorescence detection.[3] Fluorescence can provide very sensitive monitoring, but this technique suffers from several drawbacks associated with the use of photonic excitation to promote fluorescent moieties to their emitting excited state: susceptibility to photobleaching of commonly used organic fluorophores, background interferences due to Raman and Rayleigh scatterings and high levels of background fluorescence when analysing cellular extracts or imaging enzyme activities *in vivo*. Thus, luminescence technologies which require no light excitation source are becoming extremely attractive for reaching ultra-low detection limits for the targeted analytes, and result from significantly improved signal-to-noise ratios thanks to the reduction or suppression of autofluorescence issues.[4] For these reasons, during the past half-century, considerable research effort has been devoted to the understanding and applications of bioluminescence, a luminescence phenomenon triggered by a biochemical reaction within a living organism.[5] Despite many problems associated with the cell penetration of the probes, bioluminescence is now widely used as a biochemical and diagnostic tool, especially in the context of emerging and challenging biomedical applications such as *in vitro* and *in vivo* imaging.[6]

The implementation of this molecular detection technique requires the use of exogenous enzymes (*e.g.*, aequorin or the different classes of luciferase)[7,8] which are produced in various organisms through genetic engineering. However, the need to produce, handle and target luminescent proteins, and to co-localise the corresponding modified luminescent substrate (generally a modified luciferin), complicates the design of simple and reliable bioluminescent enzyme assays. To circumvent this problem, chemiluminescence appears to be a good alternative since the same advantages as bioluminescence could be obtained without the need for genetically encoded enzymes. Among the chemiluminescent species already reported, the most famous are luminol and oxalate esters because of their usual applications in forensic science (for the detection of blood) and in "light stick devices" respectively (Fig. 1A).[9] However, these chemiluminescent species require ROS (reactive oxygen species) such as hydrogen peroxide for the triggering of light emission, which is a severe limitation for their wider use in biological systems. Thus, the use of other high-energy molecules such as 1,2-dioxetanes **1**, whose luminescence can be simply triggered by the cleavage of a chemical bond under mild conditions, appears to be an excellent alternative strategy to consider for biological applications.[7,10] Indeed, the chemical or enzymatic cleavage of **1** results in the release of an aromatic anion **2** which undergoes an electron transfer according to a CIEEL (Chemically Initiated Electron Exchange Luminescence) mechanism.[11] This process results in the formation of the ester **3** in the excited state which is responsible for the light emission when returning back to the ground state (Fig. 1B).[12] Such reporter probes using 1,2-dioxetane moieties have already been used for the detection of biologically relevant enzymes such as alkaline phosphatase,[13] neuramidinase,[14] acetylcholine esterase[15]

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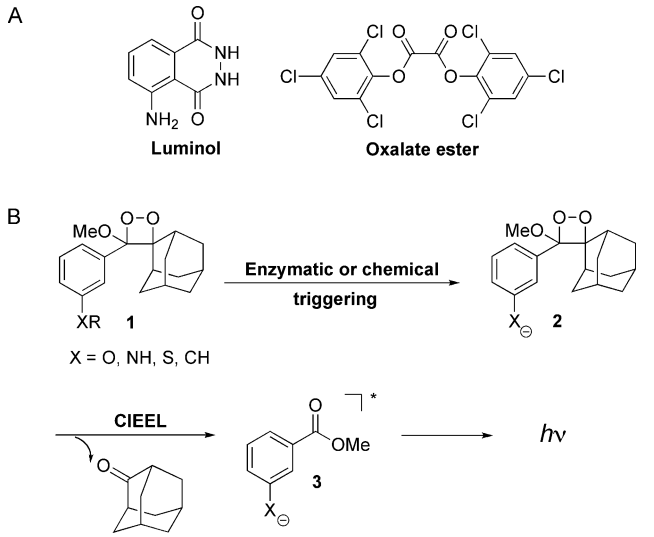


Fig. 1 Chemical structure of the main compounds currently used as chemiluminescent labels. A) Compounds chemically activated by basic hydrogen peroxide; B) CIEEL-type 1,2-dioxetanes.

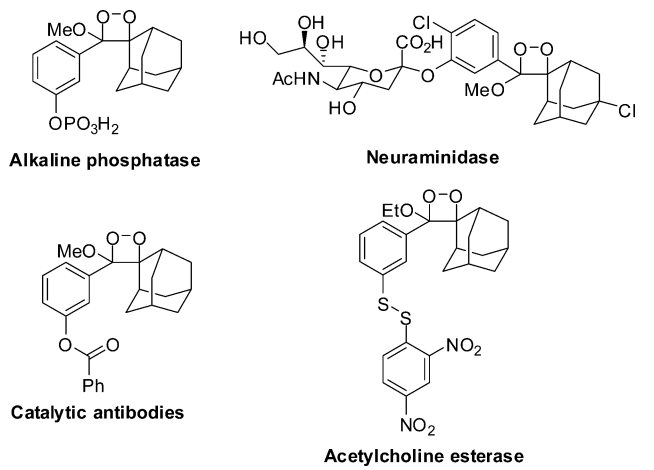


Fig. 2 Representative 1,2-dioxetane-based chemiluminescent substrates used for detecting enzyme activities.

or catalytic antibodies[16] (Fig. 2). In order to widen the scope of such chemiluminescent probes, our interest was focused on the development of chemiluminescent assays for new classes of enzymes, and we chose to focus our first efforts on proteases.

Thus, we investigated the use of such 1,2-dioxetanes as luminescent markers of peptide bond cleavage events. CIEEL-active dioxetanes bearing an aryl moiety *meta*-substituted with an amide have been described only in a patent application for the detection of peptidases. However, as the resulting aniline released is not negatively charged under physiological conditions, the electron transfer process is poorly efficient and the light emitted is particularly low (Fig. 3A).[17] Thanks to their lower p*K*a, phenolic or thiophenolic 1,2-dioxetanes are the most suitable energy sources for such enzyme-sensitive chemiluminescent bioprobes. Therefore, we decided to use phenolic 1,2-dioxetanes to develop a general method for the detection of proteases by chemiluminescence. In order to transfer the information between the enzymatic reaction itself (namely, the cleavage of an amide bond releasing an amine and a carboxylic acid), to the release of a phenol, we choose to use a self-cleavable linker (also named a self-immolative linker or reactive chemical adaptor). This self-cleavable linker will be bound to the peptidyl substrate at one end and to the dioxetane at the other. The amide bond cleavage should thus lead to a spontaneous decomposition of the linker yielding the free phenolate (Fig. 3B).[18,19] Three years ago, we patented our first results concerning the development of self-cleavable chemiluminescent substrates for protease assays,[20] and such chemical adaptor strategy has since been applied by other groups to the bioluminescent substrate luciferin for designing alkaline phosphatase-sensitive bioluminogenic substrates[21] and for the construction of releasable luciferin-transporter conjugates used as tools for the real-time analysis of cellular uptake in the context of drug delivery.[22] However, those assays all require the ancillary addition of luciferase enzyme. We recently reported our preliminary results on chemiluminescent species based on phenolic 1,2-dioxetanes;[23] reported here is the full account of this study, focused on the choice of the self-cleavable linker and implications of the chosen spacer-technology in the design of a viable and straightforward synthetic route leading to protease-sensitive chemiluminescent molecular systems.

Results and discussion

Choice of the targeted enzymes

In order to design a new general method to detect proteases through chemiluminescence, two amide bond cleaving enzymes were targeted. Firstly, penicillin amidase (also called penicillin G acylase, PGA) was chosen because this enzyme is able to recognise and cleave the simple phenylacetyl moiety and thus constitutes an interesting amine releasing enzyme for the choice of the self-cleavable linker. Furthermore, this enzyme is inexpensive and readily available in an immobilised form which tolerates the use of an organic co-solvent such as acetone or methanol. Therefore, the first model chemiluminescent probes with unoptimised properties, such as water solubility, could be rapidly evaluated. Afterwards, extension to caspase-3 was envisaged in order to apply the strategy to a representative and biologically significant protease, and open new perspectives for biological applications. Indeed, caspases are Cysteine-Aspartic-acid-ProteASEs that play a critical role in the apoptotic cascade (programmed cell death). Caspase-3 has been specifically identified as being a key mediator of apoptosis in mammalian cells:[24] activation of caspase-3 indicates that the apoptotic pathway has progressed to an irreversible stage. Disorders in the apoptotic process are involved in many diseases or disorders such as ischemia, neurodegenerative diseases or cancers. This protease is thus an attractive target for diagnostic and therapeutic applications through its detection in biological media using smart optical bioprobes. The main strategy for both *in vitro* and *in cellulo* sensing of caspase-3 relies on the use of fluorogenic probes based either on the Fluorescence Resonance Energy Transfer (FRET) or pro-fluorescence processes.[19,25] All these probes are based on the fact that upon binding with caspase-3, protease-mediated cleavage of their peptide substrate occurs after the aspartic acid residue at the C-terminal side. The preferred sequence for caspase-3 cleavage being Asp-Glu-Val-Asp.

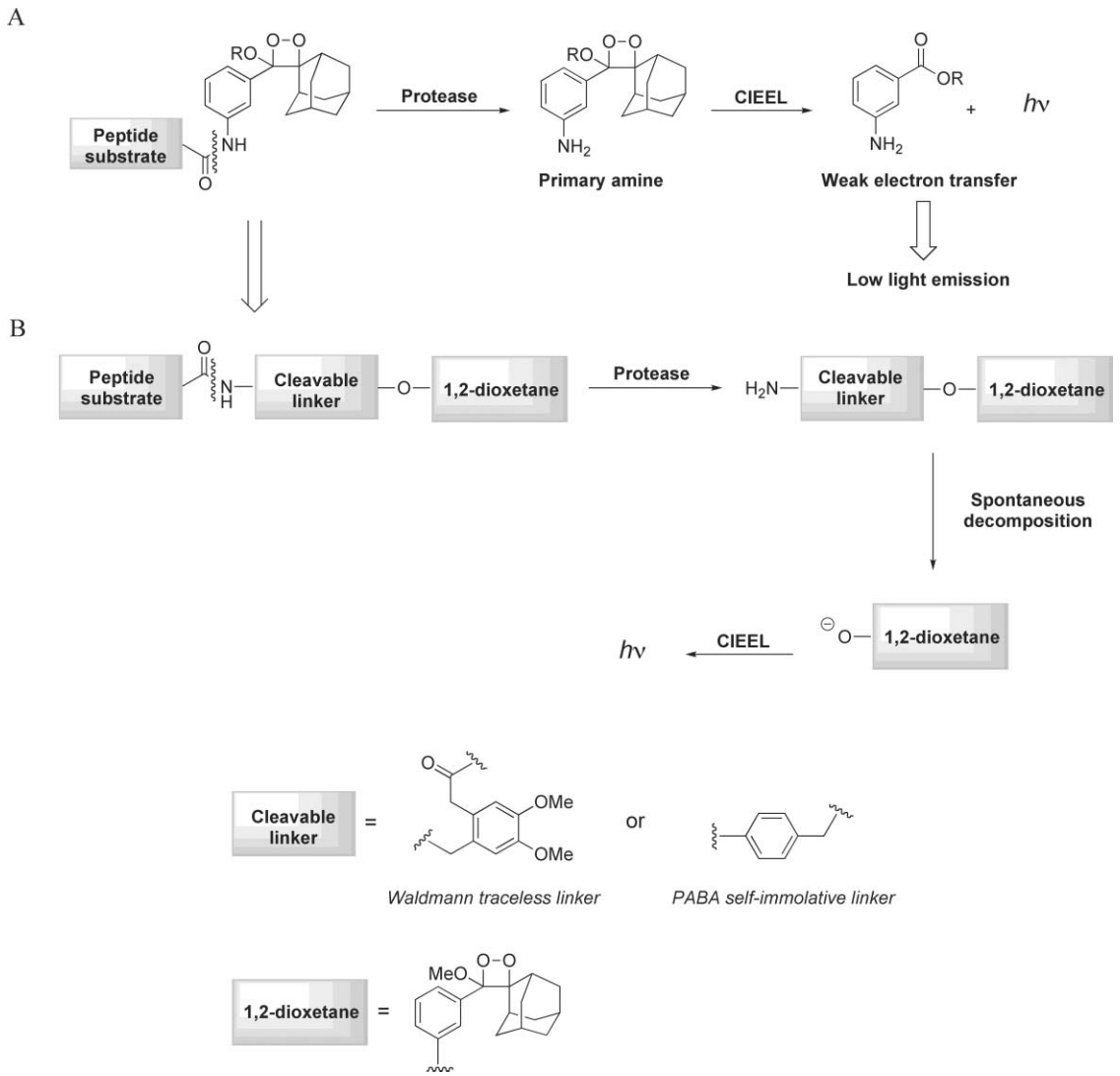


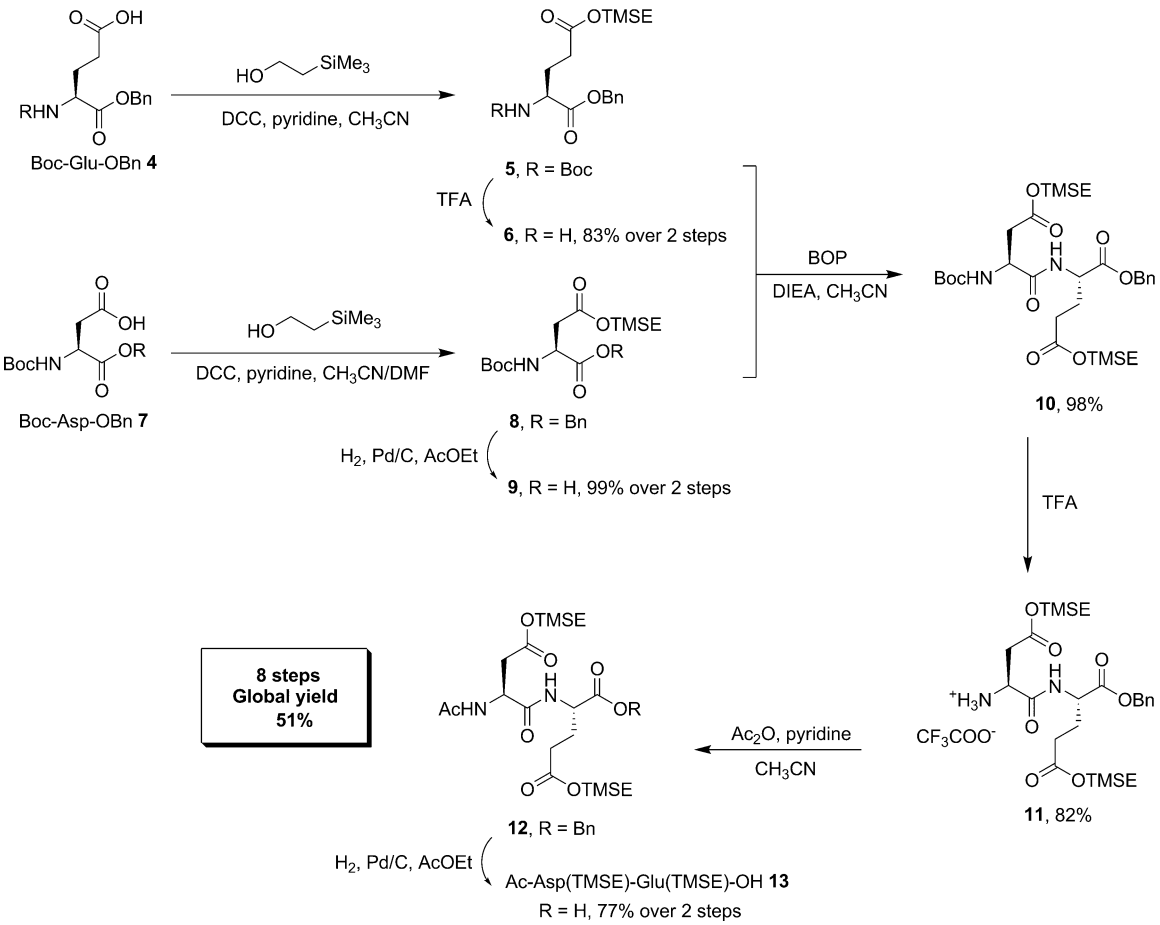
Fig. 3 General mechanisms of the activation of chemiluminescent substrates by proteolytic enzymes. A) Release of aniline-based 1,2-dioxetanes having poor light-emitting efficiency; B) release of light-emitting phenolate-based 1,2-dioxetanes through a self-immolative strategy using either Waldmann traceless linker or PABA linker.

Synthesis of the amide and peptide substrates

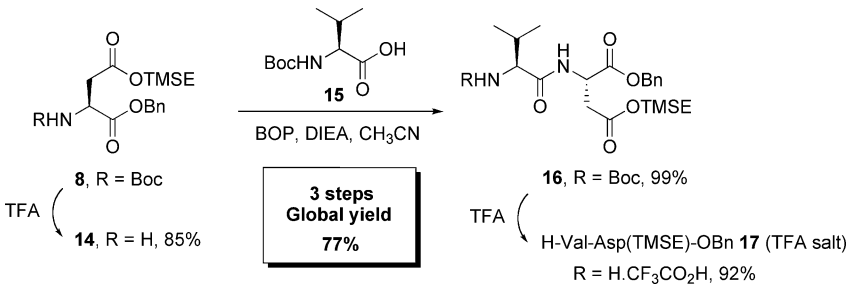
The synthesis of the PGA substrate was straightforward since commercially available phenylacetyl chloride could directly react with the amino group of the chosen self-cleavable linker (*vide infra*) to afford a carboxamide derivative suitable for the subsequent introduction of the 1,2-dioxetane moiety. More challenging was the synthesis of the caspase-3 peptidyl substrate, since an orthogonal protection of the carboxylic acid side-chains towards both the backbone amine and carboxylic acid functions is required. A solid-phase peptide synthesis using a Fmoc/*t*Bu strategy[26] could be envisioned but was hampered by the problematic Asp-Glu sequence which is prone to form undesired aspartimide derivatives especially during the Fmoc removal step.[27] A solution peptide synthesis using the Boc/benzyl strategy was thus preferred, both to avoid this tricky issue and to get the side-chain protected tetrapeptide in a multigram scale which could be further derivatised through a multi-step synthetic scheme enabling the versatile introduction of self-cleavable linkers and 1,2-dioxetane units. A crucial aspect for the synthesis of this tetrapeptide was the choice of a suitable side-chain protecting group for the aspartic and glutamic acids. It must be stable to the acidic and basic conditions encountered during the synthetic process of peptide chain elongation and also removable under mild conditions (especially as far as the pH range is concerned), since it needs to be removed at the very end of the synthesis without destruction of the self-cleavable linker. It should also be compatible with the 1,2-dioxetane ring formation, usually undertaken through [2 + 2] cycloaddition. Indeed, it is well known that 1,2-dioxetanes are sensitive to acidic media and we suspected that the linkage connecting the phenol moiety to the self-cleavable linker within the targeted chemiluminescent probes would be not fully stable under basic conditions. Thus, we chose to use 2-(trimethylsilyl)ethyl esters (TMSE),[28] since they are described to be stable enough to undergo the multi-step peptide synthesis

and could be removed by treatment with a fluoride ion source under mild conditions,[29] which have already been used with 1,2-dioxetanes.

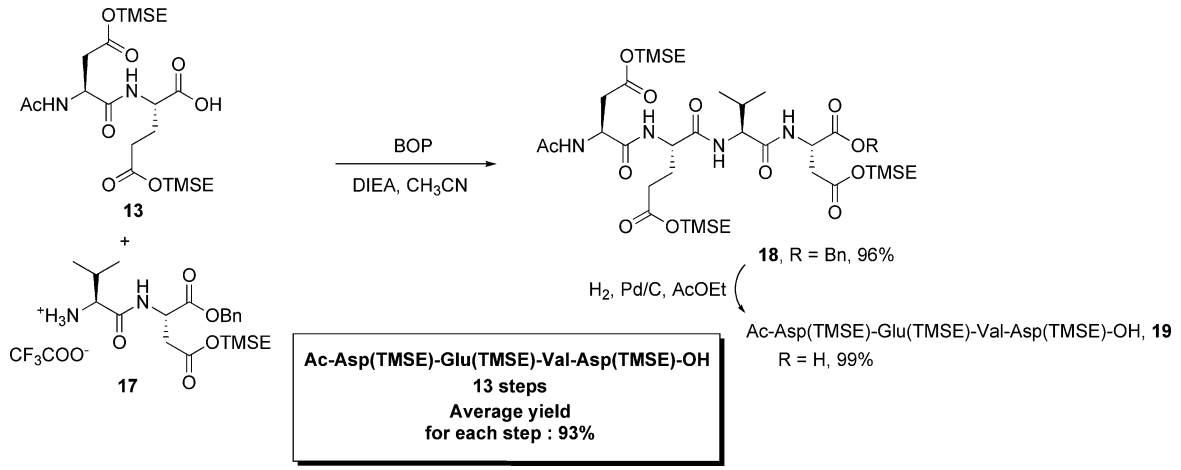
Thus, a highly convergent solution peptide synthesis was performed starting from commercially available Boc-Glu-OBn **4**, Boc-Asp-OBn **7** and Boc-Val-OH **15** (Schemes 1–3). The carboxylic acid functionalities of the side-chains were protected as TMSE esters by treatment with 2-trimethylsilylethanol and DCC, in the presence of pyridine, to give **5** and **8** in quantitative yield.[30] Removal of the Boc group of **5** by treatment with TFA and the benzyl group of **8** under standard Pd/C hydrogenolysis conditions afforded H-Glu(TMSE)-OBn **6** and Boc-Asp(TMSE)-OH **9** respectively in good yields. These two amino acid building blocks were coupled together using BOP phosphonium salt in the presence of DIEA,[31] to yield the dipeptide **10** in quantitative yield. At this stage, the Boc protecting group was removed and the *N*-terminal amino group of the dipeptide was converted into acetamide to protect the resulting caspase-3 substrate against endogenous exopeptidases. It is noteworthy that it was essential to isolate **11** as an ammonium salt to avoid an undesired cyclisation side-reaction leading to the formation of diketopiperazine. Finally, the deprotection of the benzyl ester allowed the obtention of the first dipeptide Ac-Asp(TMSE)-Glu(TMSE)-OH **13** in 51% overall yield for the 8 steps. The second dipeptide was obtained from **8** through a shorter synthetic route of sequential Boc removal, BOP-mediated peptide coupling with commercially available Boc-Val-OH **15** and Boc removal to afford ammonium **17** in 77% overall yield. The two dipeptides



Scheme 1 Preparation of dipeptide Ac-Asp(TMSE)-Glu(TMSE)-OH **13**.



Scheme 2 Preparation of dipeptide H-Val-Asp(TMSE)-OH **17**.

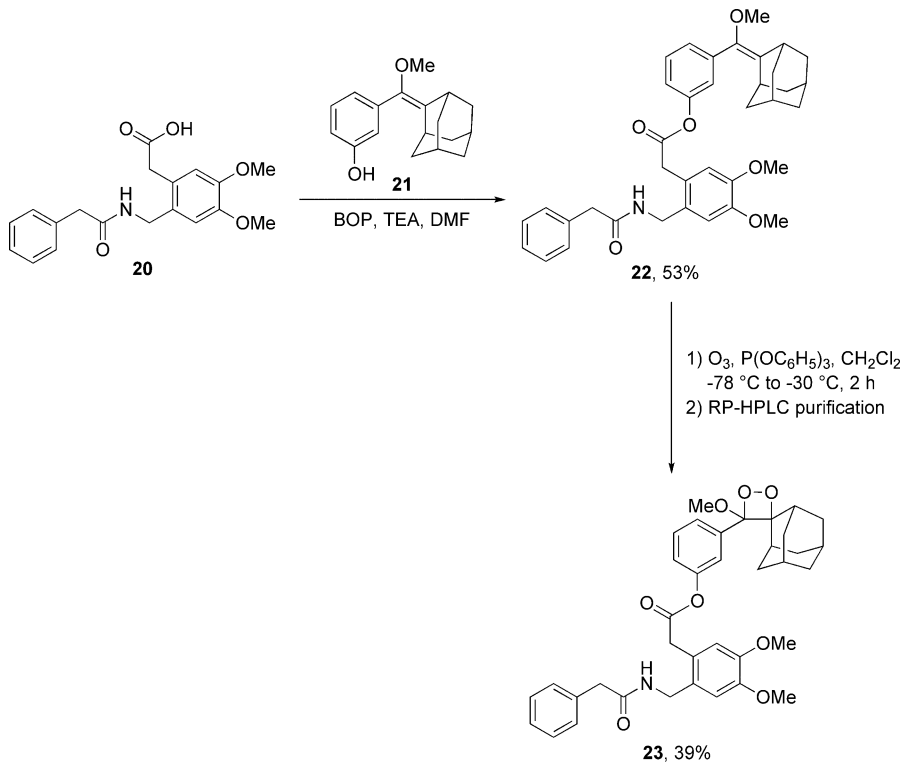


Scheme 3 Preparation of side-chain protected tetrapeptide Ac-Asp(TMSE)-Glu(TMSE)-Val-Asp(TMSE)-OH **19**.

Ac-Asp(TMSE)-Glu(TMSE)-OH **13** and H-Val-Asp(TMSE)-OBn **17** (TFA salt) were then assembled by using BOP/DIEA, affording the expected tetrapeptide Ac-Asp(TMSE)-Glu(TMSE)-Val-Asp(TMSE)-OH **19** after final Pd/C hydrogenolysis to remove the benzyl ester. The side-chain protected tetrapetide Asp-Glu-Val-Asp substrate of caspase-3 was thus obtained through an efficient and convergent 13-step route with a 93% average yield for each step.

Choice of the self-cleavable linker and its introduction onto the peptide scaffold. Synthesis of the chemiluminescent probes

Among the numerous self-cleavable linkers already described especially in the context of pro-drug design,[32] we first chose to investigate the ingenious traceless linker recently developed by Waldmann and Grether for solid-phase organic synthesis applications (Fig. 3B).[33] Indeed, this enzyme-labile safety catch linker allows the release of alcohols or amines through an original enzyme-initiated lactam-cyclisation. First, the conjugation of the phenylacetyl moiety to a phenolic 1,2-dioxetane moiety by means of this reactive chemical adaptor was explored, with the aim of synthesising a chemiluminescent probe for the detection of PGA (Scheme 4). Phenylacetamide **20**, readily prepared in 4 steps from homovanillic acid, was coupled to phenolic enol ether **21**, a precursor of the targeted 1,2-dioxetane, in the presence of BOP/TEA[34] to give the aryl ester **22** in 53% yield. Thereafter, the key step of the synthesis, the [2 + 2] cycloaddition of singlet oxygen with the enol ether moiety of **22**, was performed. $^1O_2$ was generated



Scheme 4 Preparation of PGA chemiluminescent probe **23**.

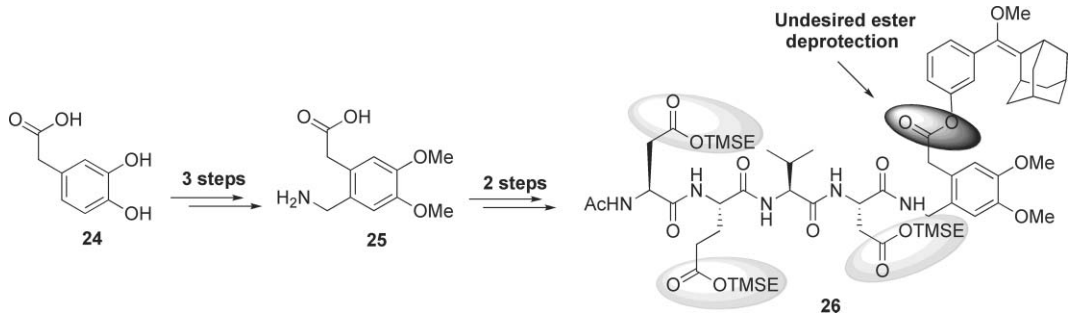


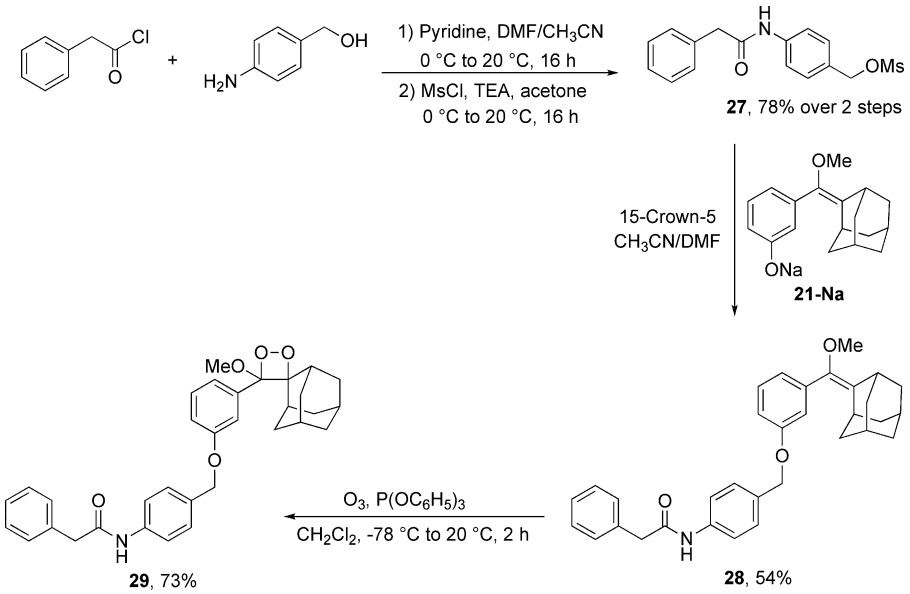
Fig. 4 Synthetic strategy used for the preparation of caspase-3 chemiluminescent probe based on the Waldmann enzyme-labile safety catch linker.

under mild conditions through a method involving reduction of ozone with triphenylphosphite.[35] Such unusual conditions were preferred to standard type II photosensitisation (*i.e.*, oxygen, visible light and a photosensitiser such as methylene blue, rose bengal or TPP)[36] as the amount of $^1O_2$ generated could be checked more carefully, thus avoiding side reactions. The resulting 1,2-dioxetane **23** was found to be stable on silica gel but purification by semi-preparative RP-HPLC was preferred to resolve the complex reaction mixture containing **23**, triphenylphosphate and some minor side-products resulting from premature degradation of the dioxetane. The structure of **23** was confirmed by ESI mass spectrometry.

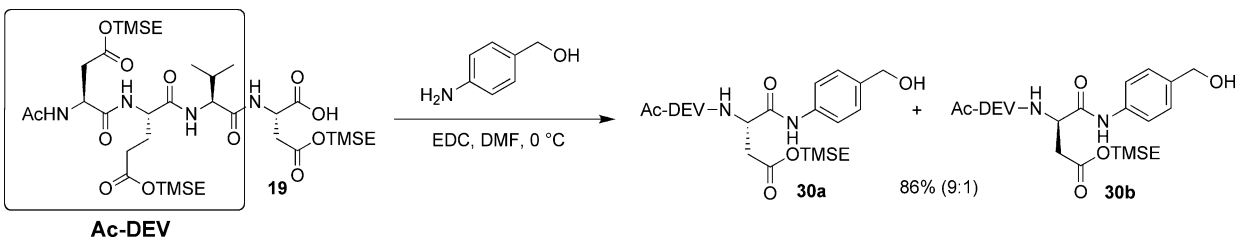
This first success in the synthesis of a self-cleavable chemiluminescent probe led us to envisage the extension of the chosen synthetic strategy to the more functionalised peptidyl substrate of caspase-3 (Fig. 4). Thus, starting from 3,4-dihydroxyphenylacetic acid **24**, amino acid **25** was efficiently synthesised in three steps and introduced onto the C-terminal carboxylic acid of tetrapeptide **19**. Further derivatisation of the resulting peptide–linker conjugate with the phenolic enol ether **21**, precursor of the targeted 1,2-dioxetane, through the BOP-mediated esterification reaction led to compound **26**. At this stage, we tested the deprotection conditions for the TMSE esters in order to check the selectivity of their removal towards the undesired hydrolysis of the aryl ester bond between the linker and the enol ether moiety. Unfortunately, in our hands all our efforts to remove the TMSE esters ended up with a simultaneous release of enol ether **21**. In addition, acidic fluoride ion sources such as TEA·3HF, HF·pyridine or TBAF buffered with PTSA[37] were not effective at inducing the deprotection of TMSE esters. Basic fluoride ion sources such as TBAF (1.0 M in THF) or TAS-F[38] were effective at obtaining an efficient removal of the TMSE esters but were accompanied by a quantitative release of enol ether **21** in the reaction mixture. The use of the Waldmann safety catch linker and of other associated reactive linkers implying amine cyclisation onto an ester linkage thus appeared incompatible with the use of TMSE protected polypeptides. Other orthogonal protecting groups could have been used, such as allyl esters, but they are sensitive to $^1O_2$ [2 + 2] cycloaddition conditions.

Thus, also taking into account the potential fragility of ester linkages in the context of *in cellulo* and *in vivo* applications, we have chosen to replace this "cyclisation" based self-cleavable linker by an "elimination" based self-cleavable linker such as *para*-aminobenzylic alcohol (PABA), a linker widely used in pro-drug strategies.[32] In addition, this spacer enables the design of tripartate pro-drugs which are readily activated under physiological conditions by enzymatic cleavage through an efficient azaquinone-methide 1,6-elimination process.[39] The potential use of PABA as a suitable reactive chemical adaptor for self-cleavable chemiluminescent probes was first evaluated through the synthesis of a PGA-sensitive chemiluminescent substrate (Scheme 5). PABA reacted with phenylacetyl chloride and the resulting benzylic alcohol was activated as the mesylate ester **27** as previously described.[19] Then, etherification with sodium salt of phenol **21** was performed and enol ether **28** could be obtained in 54% yield. Finally, the [2 + 2] cycloaddition reaction was achieved under the same conditions as described for **22**. The resulting 1,2-dioxetane **29** was found to be stable enough to be purified by silica gel column chromatography in a good yield. Comparatively, this synthetic scheme and the yields obtained proved that PABA is more practical than the Waldmann linker for the design of a simple and versatile synthetic route leading to the targeted protease-sensitive chemiluminescent probes, since this heterobifunctional linker is commercially available and so 1,2-dioxetane **29** was obtained in only 4 steps and 31% overall yield.

This interesting finding led us to envisage a further extension of this synthetic strategy to the more functionalised peptidyl substrate of caspase-3. For this purpose, PABA was introduced onto the C-terminal side of tetrapeptide **19** through a peptide coupling reaction, but this synthetic process was accompanied by epimerisation at the asymmetric centre of the C-terminal aspartic acid residue (Scheme 6). Indeed, the weak nucleophilicity of the amino group of PABA was responsible for a competitive cyclisation reaction leading to the formation of stereochemically labile 5(4H)-oxazolone, which easily racemised under the coupling reaction conditions giving rise to a mixture of diastereomers **30a** and **30b**.[40] It is interesting to note that a recent publication described similar problems for the coupling of a 7-aminocoumarin fluorescent label on a Asp-Glu-Val-Asp peptide sequence.[41] The authors found that the use of HATU as a coupling reagent and TBP as a base decreased the epimerisation ratio from 75% to 3%. In our case, we found that the use of such a base had a deleterious effect on the epimerisation level (see ESI,† Table 1, entries 1–3). Surprisingly the addition of a stoichiometric amount of HOBt, an additive well-known to reduce racemisation,[42] did not improve the diastereomeric ratio in favour of **30a** (Table 1,† entries 3–4). By using EEDQ,[43] a coupling reagent which works under neutral conditions, a better diastereoisomeric ratio (80:20) was obtained (Table 1,† entry 5) but finally the best coupling conditions were found as follows: the use of EDC and a slow warming of the



Scheme 5 Preparation of PGA chemiluminescent probe **29**.

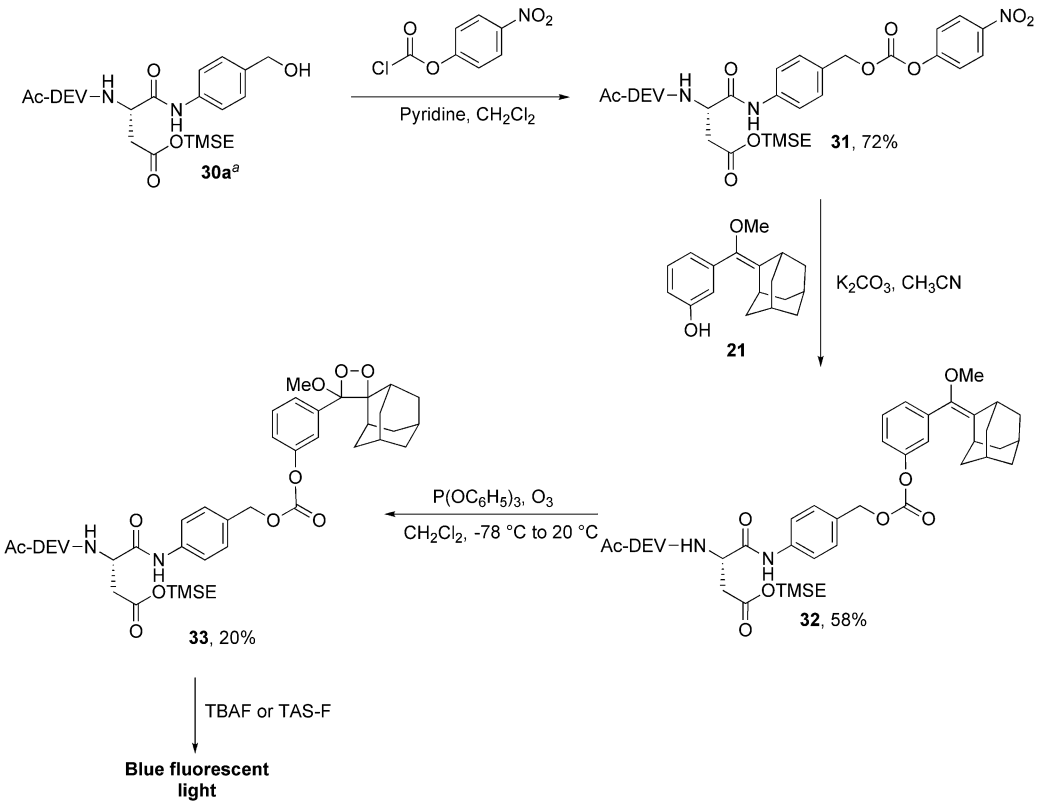


Scheme 6 Preparation of peptide–PABA conjugate **30** by EDC-mediated peptide coupling reaction.

reaction mixture from 0 °C to 20 °C (Table 1,† entry 6). In these conditions, only 10% of the undesired diastereoisomer was detected by RP-HPLC. The synthesis was taken further using the diastereoisomeric mixture and the small amount of undesired epimer **30b** was removed in the next steps (*vide infra*).

After the introduction of the PABA linker, several strategies were investigated to connect it to the phenolic enol ether **21**.[44] Knowing that the etherification reactions of peptide–PABA conjugate **27a** with phenolic compounds were often tricky and not always reproducible,[19] the first synthetic route explored was the activation of this benzylic alcohol **30a** as a *para*-nitrophenyl carbonate derivative to form a carbonate linkage with **21**.[45] Thus, enol ether **21** was reacted with activated carbonate **31** to afford **32** in 58% yield. Then, the [2 + 2] cycloaddition of $^1O_2$ to enol ether **32** was performed. Contrary to the reaction of the enol ether **28** derived from phenylacetic acid, only 50% conversion was obtained but 1,2-dioxetane **33** was isolated in a pure form by semi-preparative RP-HPLC. The removal of the undesired diastereoisomer was achieved during this chromatographic purification step. Finally, the deprotection of the TMSE esters was carried out. Unfortunately, in the presence of TBAF or TAS-F, the unstable phenolate 1,2-dioxetane **2** was released which decomposed rapidly to excited benzoate **3**, emitting a persistent blue fluorescent light which was observed in the reaction flask (Scheme 7). On the one hand, this observation was encouraging, since it confirmed that such 1,2-dioxetanes were efficient light emitters once the PABA linkage cleaved. On the other hand, it showed that the stability of the carbonate linkage was not compatible with the reaction conditions required for the removal of the TMSE groups, and could lead to a high non specific light emission.

A more stable linkage had thus to be investigated to connect the 1,2-dioxetane to the self-cleavable linker. Therefore, by analogy with the preparation of the self-cleavable PGA chemiluminescent probe **29** (*vide supra*) we decided to use again the ether function even if the synthetic yield was expected to be modest (Scheme 8). Conversion of the benzylic alcohol into the mesylate derivative **34** and its subsequent nucleophilic substitution with **21** afforded enol ether **35** in a moderate 44% overall yield for the two steps. As observed for carbonate derivative **32**, the conversion rate of the [2 + 2] cycloaddition was found to be close to 50% and 1,2-dioxetane **36** was obtained with an unoptimised 25% yield (moreover, unreacted enol ether **35** could be recovered and recycled). Finally, the complete removal of the TMSE side-chain protecting groups was successfully performed by treatment with TBAF (1.0 M in THF), highlighting the higher stability of the ether moiety compared to the previously used ester and carbonate linkages. Purification was achieved by semi-preparative RP-HPLC to give the caspase-sensitive chemiluminescent substrate **37** in quantitative yield. Importantly, for this chromatographic purification, the use of a slightly alkaline mobile phase (i.e., aq. TEAB 50 mM, pH 7.5) was found to be essential to prevent premature cleavage of the 1,2-dioxetane moiety, observed in particular with



[a]Tetrapetide **30a** was contaminated with 10% of undesired diastereomer **30b**; both enol ether and 1,2-dioxetane derived from this epimer were removed during the RP-HPLC purification of 1,2-dioxetane **33**

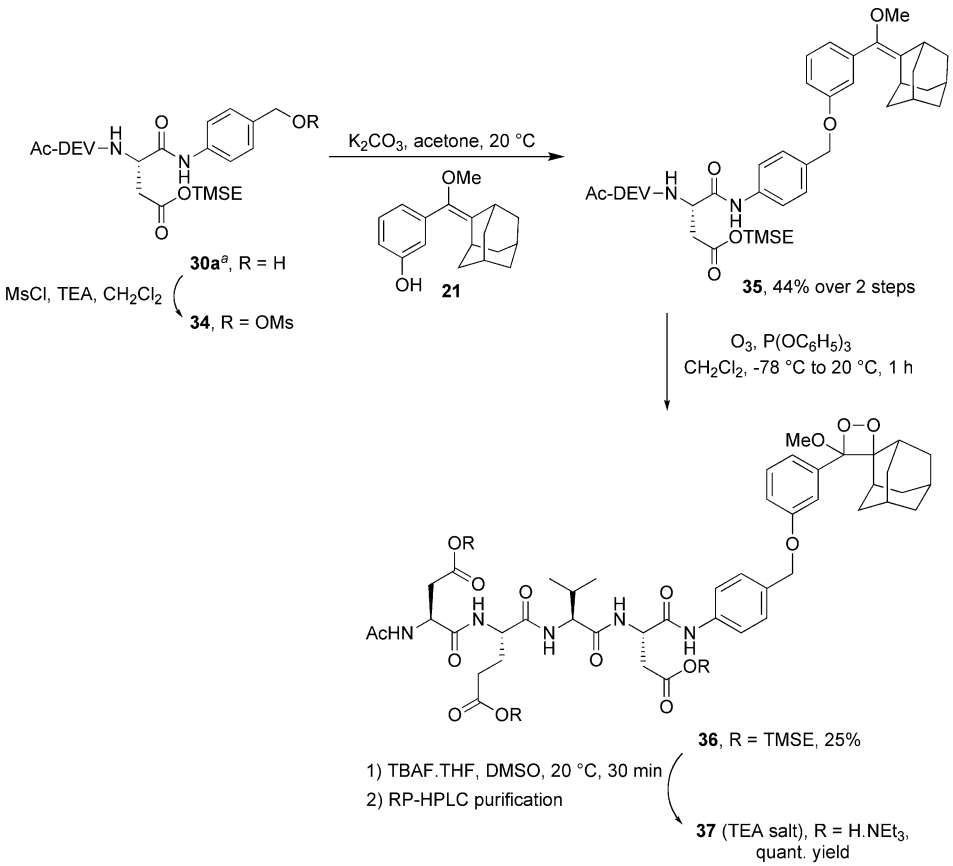
Scheme 7 Attempted preparation of the caspase-3 chemiluminescent probe bearing a carbonate linkage between PABA and the light-emitting 1,2-dioxetane moiety.

deionised water. The structure of **37** was confirmed by ESI mass spectrometry.

Characterization of chemiluminescent probes through *in vitro* protease assay

With the three self-cleavable chemiluminescent probes **23**, **29** and **37** in hand, their selective cleavage by the targeted protease (*i.e.*, PGA for **23** and **29**, and caspase-3 for **37**) was investigated. As already reported in the literature by several of us,[15] a final time detection was preferred to observe the chemiluminescence following the peptide bond cleavage event. The addition of enhancers proved critical for the observation of a significant light signal in aqueous media and so restricted the use of such chemiluminescent probes to *in vitro* assays. Thus, some additives were introduced in the enzyme buffer: a surfactant (*i.e.*, cetyltrimethylammonium bromide, CTAB) was used in order to create a hydrophobic environment (through the formation of a micelle) around the light-emitting 1,2-dioxetane moiety and a fluorophore (*i.e.*, 5-(stearoylamino)fluorescein, $\lambda_{em} = 530$ nm) to perform an energy transfer between the enzymatically released 1,2-dioxetane and the xanthene-based fluorophore.[46] Thus, after incubation of **37** with recombinant human capase-3, the light emitted was recorded and a spontaneous emission was detected around 530 nm, highlighting a complete energy transfer between **2** (normal emission at $\lambda_{em} =$ 460 nm) and the fluorophore. The luminescence rapidly increased to reach a maximum (after 90 s in the reaction conditions of Fig. 5A) and thereafter slowly decreased. It is noteworthy that even after 7.5 min, light emission still occurred. Finally, an increase in the amount of light emitted with length of enzymatic reaction time was observed, highlighting the efficiency of the enzymatic cleavage and the subsequent decomposition of the self-cleavable spacer (Fig. 5B). The same peptide cleavage reaction was performed with a decreased amount of caspase-3 introduced into the reaction buffer, and allowed us to find a detection limit of around 1.0 pmol of enzyme (Fig. 5C). Furthermore, the high degree of specificity of the cleavage was demonstrated by two control reactions in which the chemiluminescent peptide **37** was incubated with PGA and caspase-9 respectively. Indeed, in both cases, no light emission was detected (data not shown).

Chemiluminescent phenylacetamide derivatives **23** and **29** were assayed against PGA and the same experimental conditions required for unveiling the chemiluminescence of the released phenolate 1,2-dioxetane **2** in aq. buffers (*vide supra*) were used. Light emission and time-dependant changes in luminescence intensity centred at 530 nm similar to those displayed in Fig. 5A, were observed with **29**, confirming the efficiency of our strategy based on a PABA self-cleavable linker and ether linkage to detect different types of protease or related proteolytic enzymes. Furthermore, we have shown that the light emitted was different from the one generated through firefly bioluminescence. Indeed, this latter emission is known to proceed *via* multiple short-lived



[a]Tetrapetide **30a** was contaminated with 10% of undesired diastereomer **30b**; both enol ether and 1,2-dioxetane derived from this epimer were removed during the RP-HPLC purification of 1,2-dioxetane **37**

Scheme 8 Preparation of caspase-3 chemiluminescent probe **37**.

flashes of light.[7] On the contrary, the light emission observed in our case was a "glow" type chemiluminescence which is characteristic of phenolate 1,2-dioxetane **2** (see ESI,† Fig. 6). This feature is of great importance for subsequent applications since it would allow the observation of a prolonged light signal (*i.e.*, over more than 10 min) in biological media. On the other hand, we observed an anomalous behaviour of the PGA probe **23**, whose chemiluminescence was unveiled directly during incubation only with the buffer containing enhancers, and without the enzyme. This non-enzymatic activation of **23** was attributed to the release of phenolate 1,2-dioxetane **2** through the hydrolytic cleavage of its aryl ester linkage, confirming that the Waldmann linker and related self-cleavable spacers using a carboxyl functional group to graft the phenol-based chemiluminescent species to the peptidyl substrate, are not valuable candidates for our latent chemiluminophore strategy.

Conclusion

We have described the synthesis of the first self-cleavable chemiluminescent probes suitable for the detection of protease (or related proteolytic enzymes) activities. An efficient strategy was devised thanks to the use of a reactive chemical adaptor which allowed us to reconcile the detection of a protease involving a peptide bond cleavage event and the release of a light-emitting phenolic 1,2-dioxetane moiety. Our investigations have led us to identify the self-cleavable linker PABA and its connection to the 1,2-dioxetane by an ether linkage as the optimal combination for designing a reliable and robust synthetic route for these smart optical bio-probes. This flexible strategy constitutes a general method for the detection of proteolytic enzymes because a large set of fluorogenic, bioluminogenic or chemoluminogenic substrates can be produced by changing only the enzyme recognition unit or the phenol-based optical marker. It could also be easily adapted to other amine releasing enzymes. Further work is under investigation to improve the properties of chemiluminescent caspase-3 substrate **37** aimed at using it in biological media such as cellular extracts. This work is currently on-going in our laboratory and will be reported in due course.

Experimental

General

Column chromatographies were performed on silica gel (40–63 μm) from SdS. TLC were carried out on Merck DC Kieselgel 60 F-254 aluminium sheets. Compounds were visualised by one or both of the following methods: 1) staining with a 0.2% (w/v) ninhydrin solution in absolute ethanol, 2) staining with a 3.5% (w/v) phosphomolybdic acid solution in absolute ethanol. All

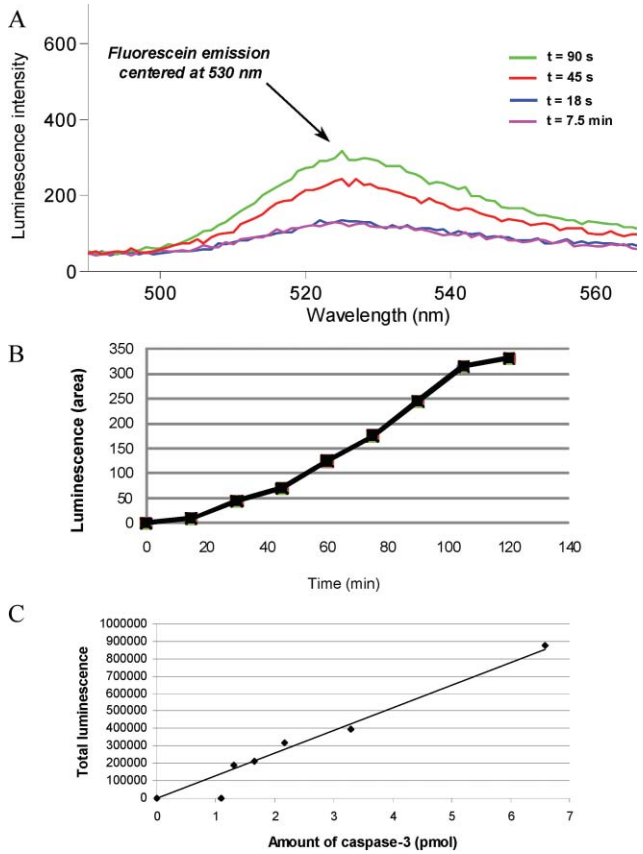


Fig. 5 *In vitro* cleavage of self-cleavable chemiluminescent probe **37** by recombinant human caspase-3. A) Chemiluminescence spectra of probe **37** (concentration: 30 μM) in buffer containing enhancers (CTAB, 2 mM, fluorescein 0.18 mM) recorded after incubation with recombinant human caspase-3 (1.6 10^{-3} U, incubation time: 105 min); B) maximum light emission of probe **37** as a function of the incubation time with recombinant human caspase-3; C) determination of the detection limit of caspase-3: light emission could be observed until a caspase-3 amount of 1.31 pmol.

solvents were dried following standard procedures (acetonitrile: distillation over CaH_2, dichloromethane: distillation over P_2O_5, DMF: distillation over BaO under reduced pressure). DIEA, pyridine and triethylamine were distilled from CaH_2 and stored over BaO. Phenolic enol ether **21** derived from adamantanone was synthesised using literature procedures[44] and preparation of phenylacetamide **20** and mesylate esters **27** and **34** was recently described by us.[19,20] Recombinant human caspase-3 and 9 enzymes (respectively 5.52 U/mg and 7139.39 U/mg) were purchased from Sigma. Recombinant *A. faecalis* PGA (0.63 U/mg) was gratefully furnished by Pr. L. Fischer, Universität Hohenheim (Institut für Lebensmitteltechnologie, Fachgebiet Biotechnologie, Stuttgart). The HPLC-grade acetone and acetonitrile (CH_3CN) were obtained from Acros and Panreac respectively. Buffers (caspase-3, caspase-9, alkaline and phosphate buffers) and aq. mobile phases for HPLC were prepared using deionised water purified with a Milli-Q system (purified to 18.2 MΩ.cm). Triethylammonium acetate (TEAA, 1.0 M) and triethylammonium bicarbonate (TEAB, 1.0 M) buffers were prepared from distilled triethylamine and glacial acetic acid and CO_2 gas respectively. ^1H and ^{13}C NMR spectra were recorded on a Bruker DPX 300 spectrometer (Bruker, Wissembourg, France). Chemical shifts are expressed in parts per million (ppm) from $CDCl_3$ ($\delta_H = 7.26$, $\delta_C = 77.36$) or CD_3OD ($\delta_H = 3.34$, $\delta_C = 49.86$).[47] J values are expressed in Hz. Optical rotations were measured with a Perkin Elmer 341 polarimeter. Infrared (IR) spectra were recorded as thin-film on sodium chloride plates or KBr pellets using a Perkin Elmer FT-IR Paragon 500 spectrometer. UV–visible spectra were obtained on a Varian Cary 50 scan spectrophotometer. Chemiluminescence spectroscopic studies were performed with a Varian Cary Eclipse spectrophotometer. Analytical HPLC was performed on a Thermo Electron Surveyor instrument equipped with a PDA detector. Semi-preparative HPLC was performed on a Finnigan SpectraSYSTEM liquid chromatography system equipped with UV–visible 2000 detector. Mass spectra were obtained either with a Finnigan LCQ Advantage MAX (ion trap) apparatus equipped with an electrospray source or by MALDI-TOF mass spectrometry on a Voyager DE PRO in the reflector mode with α-CHCA as a matrix.

High-performance liquid chromatography separations

Several chromatographic systems were used for the analytical experiments and the purification steps. **System A**: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 4.6 × 150 mm) with CH_3CN and triethylammonium acetate (TEAA, 100 mM, pH 7.0) as the eluents [75% TEAA (2 min), then linear gradient from 25 to 100% (30 min) of CH_3CN] at a flow rate of 1.0 mL min^{-1}. Dual UV–visible detection was achieved at 254 and 285 nm. **System B**: RP-HPLC (Macherey-Nagel Nucleosil C_{18} column, 5 μm, 10 × 250 mm) with CH_3CN and deionised water as the eluents [75% H_2O (5 min), then linear gradient from 25 to 55% (15 min) and 55 to 90% (70 min) of CH_3CN] at a flow rate of 5.0 mL min^{-1}. UV detection was achieved at 260 nm. **System C**: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 4.6 × 150 mm) with CH_3CN and deionised water as the eluents [100% H_2O (5 min), then linear gradient from 0 to 100% (40 min) of CH_3CN] at a flow rate of 1.0 mL min^{-1}. Dual UV–visible detection was achieved at 210 and 254 nm. **System D**: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 10 × 250 mm) with CH_3CN and TFA 0.1% as the eluents [80% TFA (5 min), then linear gradient from 20 to 68% (20 min), 68 to 80% (10 min) and 80 to 100% (5 min) of CH_3CN] at a flow rate of 5.0 mL min^{-1}. Dual UV–visible detection was achieved at 210 and 250 nm. **System E**: system C with the following gradient [75% H_2O (2 min), then linear gradient from 25 to 100% (30 min) of CH_3CN]. **System F**: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 10 × 250 mm) with CH_3CN and deionised water as the eluents [75% H_2O (3 min), then linear gradient from 25 to 65% (15 min) and 65 to 100% (60 min) of CH_3CN] at a flow rate of 5.0 mL min^{-1}. Dual UV–visible detection was achieved at 210 and 250 nm. **System G**: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 10 × 250 mm) with CH_3CN and triethylammonium bicarbonate (TEAB, 50 mM, pH 7.5) as the eluents [100% TEAB (5 min), then linear gradient from 0 to 100% (65 min) of CH_3CN] at a flow rate of 4.0 mL min^{-1}. Dual UV–visible detection was achieved at 210 and 250 nm.

General procedure for the side chain protection of glutamic and aspartic acids Boc-Glu(TMSE)-OBn (5) and Boc-Asp(TMSE)-OBn (8). Boc-Glu-OBn or Boc-Asp-OBn was dissolved in dry CH_3CN (0.8 M) and dry DMF was added until complete solubilisation. To this solution was added dry pyridine (2 equiv)

and 2-(trimethylsilyl)ethanol (1.2 equiv). The mixture was cooled to 0 °C, then DCC (1.1 equiv) was added and the resulting reaction mixture was stirred for 16 h at room temperature. Oxalic acid (5.0 M in DMF) was added and the mixture was stirred for 20 min. The DCU precipitate was filtered, washed with AcOEt and the combined filtrates were evaporated to dryness. The resulting oily residue was dissolved in AcOEt and washed successively with a sat. solution of $NaHCO_3$, deionised water, aq. citric acid (10%) and again with deionised water. The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel (CH_2Cl_2–AcOEt, 95 : 5, v/v) for **5** and pure CH_2Cl_2 for **8**, yielding the corresponding TMSE ester.

Boc-Glu(TMSE)-OBn **5** (colorless oil, quantitative yield): R_f (CH_2Cl_2–AcOEt, 95 : 5, v/v) 0.5; $[\alpha]^{21}_{365}$ +4.6° (*c* 1.12 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.03 (s, 9H, Si(CH_3)$_3$), 0.95 (t, J = 7.0 Hz, 2H, CH_2Si), 1.43 (s, 9H, C(CH_3)$_3$), 1.91–1.96 (m, 1H, 0.5CH_2), 2.15–2.19 (m, 1H, 0.5CH_2), 2.27–2.42 (m, 2H, CH_2CO), 4.14 (t, J = 7.0 Hz, 2H, OCH_2), 4.35–4.41 (m, 1H, CH), 5.10 (d, J = 9.0 Hz, 1H, NH), 5.17 (s, 2H, ArCH_2O), 7.35 (s, 5H, 5ArH); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ –1.3 (3C), 17.5, 27.9, 28.5 (3C), 30.7, 53.3, 63.1, 67.4, 80.2, 128.5–128.9 (3 peaks, 5C), 135.5, 155.6, 172.4, 173.0; IR (neat): ν_{max} 3371, 2955, 2899, 1732, 1500, 1455, 1391, 1367, 1251, 1169, 1049; MS (ESI+): *m/z* 460.27 [M + Na]$^+$, calcd for [$C_{22}H_{35}NO_6SiNa$]$^+$ 460.21; elemental analysis: calcd (%) for $C_{22}H_{35}NO_6Si$: C, 60.38; H, 8.06; N, 3.20; found: C, 60.36; H, 8.02; N, 3.44.

Boc-Asp(TMSE)-OBn **8** (colorless oil, quantitative yield): R_f (CH_2Cl_2) 0.5; $[\alpha]^{21}_{365}$ +12.7° (*c* 0.98 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.02 (s, 9H, Si(CH_3)$_3$), 0.93 (t, J = 9.0 Hz, 2H, CH_2Si), 1.43 (s, 9H, C(CH_3)$_3$), 2.79 (dd, J = 4.5 and 17.0 Hz, 1H, 0.5CH_2), 3.00 (dd, J = 4.5 and 17.0 Hz, 1H, 0.5CH_2), 4.11 (t, J = 9.0 Hz, 2H, OCH_2), 4.57–4.63 (m, 1H, CH), 5.14 (Abd, J = 12 Hz, 1H, 0.5ArCH_2O), 5.22 (Abd, J = 12.0 Hz, 1H, 0.5ArCH_2O), 5.52 (d, J = 9.0 Hz, 1H, NH), 7.34 (s, 5H, 5ArH); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ –1.2 (3C), 17.5, 28.6 (3C), 37.2, 50.4, 63.7, 67.7, 80.4, 128.5–128.8 (3 peaks, 5C), 135.6, 155.7, 171.3 (2C); IR (neat): ν_{max} 3376, 2955, 2899, 1724, 1499, 1456, 1412, 1391, 1367, 1342, 1251, 1169, 1046; MS (ESI+): *m/z* 446.33 [M + Na]$^+$, calcd for [$C_{21}H_{33}NO_6SiNa$]$^+$ 446.20; elemental analysis: calcd (%) for $C_{21}H_{33}NO_6Si$: C, 59.55; H, 7.85; N, 3.31; found: C, 59.41; H, 7.76; N, 3.33.

General procedure for the removal of Boc protecting groups. Amino acid **5** (or **8**) or dipeptide **10** (or **16**) building block was dissolved in dry CH_2Cl_2 (0.1 M). The solution was cooled to 0 °C and TFA (20 equiv) was added. The reaction mixture was stirred at room temperature for 2 h. Then, the reaction mixture was cooled to 0 °C and a solution of sat. $NaHCO_3$ was added. The aqueous layer was extracted twice with CH_2Cl_2 and the combined organic layers were washed with deionised water, dried over Na_2SO_4, filtered and concentrated under reduced pressure to afford the corresponding primary amine (for the dipeptides **11** and **17**, 1.2 equiv of TFA was added to form the corresponding ammonium salt).

H-Glu(TMSE)-OBn **6** (yellow oil, 84%): R_f (CH_2Cl_2–AcOEt, 1 : 1, v/v) 0.5; $[\alpha]^{21}_{365}$ –2.4° (*c* 1.12 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.03 (s, 9H, Si(CH_3)$_3$), 0.97 (t, J = 8.0 Hz, 2H, CH_2Si), 1.51 (s, 2H, NH_2), 1.81–1.91 (m, 1H, 0.5CH_2), 2.04–2.14 (m, 1H, 0.5CH_2), 2.43 (t, J = 7.5 Hz, 2H, CH_2CO), 3.49–3.54 (m, 1H, CH), 4.15 (t, J = 8.0 Hz, 2H, OCH_2), 5.15 (s, 2H, ArCH_2O), 7.35 (s, 5H, 5ArH); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ –1.2 (3C), 17.6, 30.0, 31.1, 54.2, 63.0, 67.1, 128.6–128.9 (3 peaks, 5C), 135.9, 173.6, 175.8; IR (neat): ν_{max} 3323, 3019, 2957, 2898, 1732, 1694, 1520, 1455, 1417, 1392, 1326, 1251, 1217, 1042; MS (MALDI-TOF, positive mode, α-CHCA matrix): *m/z* 338.47 [M + H]$^+$, calcd for [$C_{17}H_{28}NO_4Si$]$^+$ 338.18; elemental analysis: calcd (%) for $C_{17}H_{27}NO_4Si$: C, 60.50; H, 8.06; N, 4.15; found: C, 60.34; H, 7.89; N, 4.03.

H-Asp(TMSE)-Glu(TMSE)-OBn **11** (TFA salt, yellow oil, 82%): R_f (CH_2Cl_2–AcOEt, 1 : 1, v/v) 0.5; $[\alpha]^{21}_{365}$ +5.1° (*c* 1.01 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.02 (s, 9H, Si(CH_3)$_3$), 0.03 (s, 9H, Si(CH_3)$_3$), 0.91–1.02 (m, 4H, 2CH_2Si), 1.97–2.23 (m, 2H, CH_{2Glu}), 2.33 (t, J = 7.0 Hz, 2H, CH_{2Glu}CO), 2.93 (d, J = 7.0 Hz, 2H, CH_{2Asp}), 4.10–4.24 (m, 4H, 2OCH_2), 4.49 (t, J = 7.0 Hz, 1H, C*H_{Asp}), 4.52–4.60 (m, 1H, C*H_{Glu}), 5.10 (Abd, J = 12 Hz, 1H, 0.5ArCH_2O), 5.18 (Abd, J = 12 Hz, 1H, 0.5ArCH_2O), 7.33 (s, 5H, 5ArH), 7.95 (d, J = 8.0 Hz, 1H, NH_{Glu}); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ –1.4 (3C), –1.3 (3C), 17.4 (2C), 26.8, 30.5, 35.2, 50.1, 52.6, 63.8, 65.1, 67.9, 128.6–129.0 (3 peaks, 5C), 135.2, 168.6, 171.0, 171.4, 174.1; IR (neat): ν_{max} 3361, 2954, 2886, 2360, 1737, 1682, 1506, 1455, 1386, 1250, 1172, 1063; MS (MALDI-TOF, positive mode, α-CHCA matrix): *m/z* 575.46 [M + Na]$^+$, calcd for [$C_{26}H_{44}N_2O_7Si_2Na$]$^+$ 575.26.

H-Asp(TMSE)-OBn **14** (yellow oil, 85%): R_f (CH_2Cl_2–AcOEt, 1 : 1, v/v) 0.5; $[\alpha]^{21}_{365}$ –84.0° (*c* 1.01 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.02 (s, 9H, Si(CH_3)$_3$), 0.95 (t, J = 7 Hz, 2H, CH_2Si), 1.79 (s, 2H, NH_2), 2.70 (dd, J = 7.0 and 17.0 Hz, 1H, 0.5CH_2), 2.79 (dd, J = 5.0 and 17.0 Hz, 1H, 0.5CH_2), 3.82–3.86 (m, 1H, CH), 4.14 (t, J = 7.0 Hz, 2H, OCH_2), 5.16 (s, 2H, ArCH_2O), 7.35 (s, 5H, 5ArH); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ –1.2 (3C), 17.6, 39.3, 51.6, 63.4, 67.3, 128.6–128.9 (2 peaks, 5C), 135.8, 171.6, 174.5; IR (neat): ν_{max} 3196, 3065, 2954, 2897, 1735, 1681, 1498, 1455, 1250, 1172, 1042; MS (MALDI-TOF, positive mode, α-CHCA matrix): *m/z* 324.48 [M + H]$^+$, calcd for [$C_{16}H_{26}NO_4Si$]$^+$ 324.16; elemental analysis: calcd (%) for $C_{16}H_{25}NO_4Si$: C, 59.41; H, 7.79; N, 4.33; found: C, 59.34; H, 7.39; N, 4.23.

H-Val-Asp(TMSE)-OBn **17** (TFA salt, white solid, 92%): R_f (CH_2Cl_2–AcOEt, 1 : 1, v/v) 0.5; $[\alpha]^{21}_{365}$ +38.7° (*c* 0.97 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.01 (s, 9H, Si(CH_3)$_3$), 0.88–0.99 (m, 8H, CH_2Si + 2CH_{3Val}), 2.13–2.19 (m, 1H, CHCH$_3$), 2.76 (dd, J = 5.0 and 17.0 Hz, 1H, 0.5CH_2), 2.98 (dd, J = 5.0 and 17.0 Hz, 1H, 0.5CH_2), 3.85 (d, J = 5.0 Hz, 1H, C*H_{Val}), 4.05–4.13 (m, 2H, OCH_2), 4.91–4.94 (m, 1H, C*H_{Asp}), 5.09 (bd, J = 12 Hz, 1H, 0.5ArCH_2O), 5.17 (Abd, J = 12 Hz, 1H, 0.5ArCH_2O), 7.27–7.31 (m, 5H, 5ArH); 7.55 (d, J = 8.0 Hz, 1H, NH_{Asp}), 8.10 (s$_{br}$, 3H, NH_3^+); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ –1.3 (3C), 17.4, 17.8, 18.0, 30.6, 35.9, 49.1, 59.0, 64.0, 68.1, 128.8–128.9 (2 peaks, 5C), 135.3, 168.3, 170.3, 171.7; IR (KBr): ν_{max} 3366, 2947, 1671, 1542, 1429, 1385, 1353, 1288, 1180, 1138, 1055; MS (ESI+): *m/z* 423.13 [M + H]$^+$, calcd for [$C_{21}H_{35}N_2O_5Si$]$^+$ 423.23; elemental analysis: calcd (%) for $C_{23}H_{35}F_3N_2O_7Si$: C, 51.48; H, 6.57; N, 5.22; found: C, 51.51; H, 6.43; N, 5.17.

General procedure for the removal of Bn protecting groups. Amino acid **8** or peptide **12** (or **18**) building block was dissolved in AcOEt (0.06 M). The solution was cooled to 0 °C and Pd/C catalyst (10%, 40 mg for each mmol of amino acid or peptide) was added. Then, the reaction mixture was stirred under a hydrogen

atmosphere at room temperature for 16 h. Thereafter, Pd/C catalyst was removed by filtration through a Celite® 545 pad, and the filtrate was concentrated under vacuum to yield the corresponding carboxylic acid.

Boc-Asp(TMSE)-OH **9** (colorless crystals, quantitative yield): R_f (CH_2Cl_2–CH_3OH, 95 : 5, v/v) 0.4; $[\alpha]^{21}_{365}$ +106.3° (*c* 0.98 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.03 (s, 9H, Si(C*H*$_3$)$_3$), 0.98 (t, J = 7.0 Hz, 2H, C*H*$_2$Si), 1.43 (s, 9H, C(C*H*$_3$)$_3$), 2.80 (dd, J = 5.0 and 17.0 Hz, 1H, 0.5C*H*$_2$), 3.00 (dd, J = 4.0 and 17.0 Hz, 1H, C*H*$_2$), 4.18 (t, J = 7.0 Hz, 2H, OC*H*$_2$), 4.58–4.61 (m, 1H, C*H*), 5.56 (d, J = 9.0 Hz, 1H, N*H*), 11.05 (brs, 1H, COO*H*); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 1.2 (3C), 17.6, 28.6 (3C), 37.0, 50.1, 63.9, 80.7, 155.9, 171.7, 176.5; IR (KBr): ν_{max} 3445, 2956, 1736, 1658, 1519, 1456, 1412, 1393, 1371, 1355, 1335, 1289, 1252, 1219, 1196, 1175, 1066, 1048; MS (MALDI-TOF, positive mode, α-CHCA matrix): *m*/*z* 372.42 $[M + K]^+$, calcd for $[C_{14}H_{26}NO_6SiK]^+$ 372.12; elemental analysis: calcd (%) for $C_{14}H_{26}NO_6Si$: C, 50.43; H, 8.16; N, 4.20; found: C, 50.35; H, 8.13; N, 4.21.

Ac-Asp(TMSE)-Glu(TMSE)-OH **13** (colorless oil, quantitative yield): R_f (100% AcOEt) 0.2; $[\alpha]^{21}_{365}$ +32.1° (*c* 0.39 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.03 (s, 9H, Si(C*H*$_3$)$_3$), 0.06 (s, 9H, Si(C*H*$_3$)$_3$), 0.96–1.02 (m, 4H, 2C*H*$_2$Si), 1.96–2.10 (m, 4H, C*H*$_3$ + 0.5C*H*$_{2Glu}$), 2.15–2.30 (m, 1H, 0.5C*H*$_{2Glu}$), 2.32–2.51 (m, 2H, C*H*$_{2Glu}$CO), 2.63 (dd, J = 6.0 and 17.0 Hz, 1H, 0.5C*H*$_{2Asp}$), 2.98 (dd, J = 4.0 and 17.0 Hz, 1H, 0.5C*H*$_{2Asp}$), 4.09–4.22 (m, 4H, 2OC*H*$_2$), 4.46–4.53 (m, 1H, C**H*$_{Glu}$), 4.83–4.89 (m, 1H, C**H*$_{Asp}$), 7.03 (d, J = 8.0 Hz, 1H, N*H*$_{Asp}$), 7.56 (d, J = 8.0 Hz, 1H, N*H*$_{Glu}$); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ –1.17 (6C), 17.5, 17.6, 23.5, 26.8, 30.9, 36.1, 49.6, 52.6, 63.5, 64.0, 171.3, 171.4, 172.7, 173.9, 174.0; IR (neat): ν_{max} 3308, 2954, 1733, 1656, 1536, 1389, 1250, 1173, 1063; MS (MALDI-TOF, positive mode, α-CHCA matrix): *m*/*z* 527.58 $[M + Na]^+$, calcd for $[C_{21}H_{40}N_2O_8Si_2Na]^+$ 527.22; elemental analysis: calcd (%) for $C_{21}H_{40}N_2O_8Si_2$, 0.14CH_2Cl_2: C, 49.13; H, 7.86; N, 5.42; found: C, 48.87; H, 7.84; N, 5.50

Ac-Asp(TMSE)-Glu(TMSE)-Val-Asp(TMSE)-OH **19** (gray solid, quantitative yield): R_f (CH_2Cl_2–MeOH, 95 : 5, v/v) 0.5; $[\alpha]^{21}_{365}$ +17.5° (*c* 0.94 in $CHCl_3$); ^{1}H NMR (300 MHz, CD_3OD): δ 0.08 (s, 27H, 3Si(C*H*$_3$)$_3$), 0.96–1.08 (m, 12H, 3C*H*$_2$Si + 2C*H*$_{3Val}$), 1.91–2.05 (m, 4H, 0.5C*H*$_{2Glu}$ + C*H*$_3$), 2.12–2.24 (m, 2H, C*H*CH$_3$ + 0.5C*H*$_{2Glu}$), 2.38–2.50 (m, 2H, C*H*$_{2Glu}$CO), 2.65–2.93 (m, 4H, 2C*H*$_{2Asp}$), 4.16–4.28 (m, 7H, C**H*$_{Val}$ + 3OC*H*$_2$), 4.40–4.45 (m, 1H, C**H*$_{Glu}$), 4.70–4.80 (m, 2H, 2C**H*$_{Asp}$), 7.97 (d, J = 8.0 Hz, 1H, N*H*$_{Val}$), 8.22 (d, J = 8.0 Hz, 1H, N*H*$_{Asp}$), 8.32 (d, J = 8.0 Hz, 1H, N*H*$_{Asp}$), 8.41 (d, J = 8.0 Hz, 1H N*H*$_{Glu}$); ^{13}C NMR (75.4 MHz, CD_3OD): δ –0.6 (9C), 19.0 (3C), 19.6, 20.6, 23.4, 28.8, 32.3, 32.6, 37.8, 38.1, 51.2, 52.2, 55.0, 61.0, 64.7, 65.0, 65.2, 173.1, 173.3, 173.8, 173.9, 174.0, 174.2(0), 174.2(4), 175.7; IR (KBr): ν_{max} 3926, 2958, 1732, 1694, 1668, 1645, 1564, 1538, 1393, 1360, 1251, 1177, 1064; MS (MALDI-TOF, positive mode, α-CHCA matrix): *m*/*z* 841.57 $[M + Na]^+$, calcd for $[C_{35}H_{66}N_4O_{12}Si_3Na]^+$ 841.39; elemental analysis: calcd (%) for $C_{35}H_{66}N_4O_{12}Si_3$,1.2$CH_2Cl_2$: C, 47.20; H, 7.48; N, 6.08; found: C, 47.14; H, 7.22; N, 6.21.

Synthesis of Ac-Asp(TMSE)-Glu(TMSE)-OBn (12). TFA salt of H-Asp(TMSE)-Glu(TMSE)-OBn **11** (892 mg, 1.34 mmol) was dissolved in dry CH_3CN (7 mL). Then, acetic anhydride (505 μL, 5.35 mmol, 4 equiv) and dry pyridine (650 μL, 8.04 mmol, 6 equiv) were added and the resulting reaction mixture was stirred at room temperature for 1 h. Thereafter, EtOH (500 μL) was added and the mixture was stirred for 20 min in order to convert the excess of acetic anhydride to AcOEt. The reaction mixture was concentrated to dryness and the resulting oily residue was dissolved in CH_2Cl_2 (50 mL). The organic phase was successively washed with aq. citric acid (10%), deionised water, sat. $NaHCO_3$ and deionised water (20 mL each). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by chromatography on a silica gel column using CH_2Cl_2–AcOEt (7 : 3, v/v) as the mobile phase, affording Ac-Asp(TMSE)-Glu(TMSE)-OBn **12** as a colorless oil which quickly crystallized (617 mg, 77%). R_f (CH_2Cl_2–AcOEt, 7 : 3, v/v) 0.5; $[\alpha]^{21}_{436}$ +5.5° (*c* 0.95 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.03 (s, 18H, 2Si(C*H*$_3$)$_3$), 0.93–1.02 (m, 4H, 2C*H*$_2$Si), 1.90–2.04 (m, 4H, C*H*$_3$ + C*H*$_{2Glu}$), 2.1–2.4 (m, 4H, C*H*$_{2Glu}$CO + C*H*$_{2Glu}$), 2.57 (dd, J = 6.0 and 17.0 Hz, 1H, 0.5C*H*$_{2Asp}$), 2.98 (dd, J = 4.0 and 17 Hz, 1H, 0.5C*H*$_{2Asp}$), 4.11–4.21 (m, 4H, 2OC*H*$_2$), 4.52–4.62 (m, 1H, C**H*$_{Glu}$), 4.77–4.87 (m, 1H, C**H*$_{Asp}$), 5.15 (s, 2H, ArC*H*$_2$O), 6.78 (d, J = 8 Hz, 1H, N*H*$_{Asp}$), 7.34 (s, 5H, 5Ar*H*); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ –1.2 (6C), 17.5, 17.6, 23.6, 27.2, 30.6, 36.2, 49.4, 52.4, 63.4, 63.9, 67.6, 128.6–129.0 (3 peaks, 5C), 135.5, 170.4, 170.9, 171.4, 172.8, 173.3; IR (KBr): ν_{max} 3290, 3092, 2955, 2899, 1733, 1643, 1557, 1456, 1425, 1385, 1352, 1304, 1250, 1179, 1164, 1060; MS (MALDI-TOF, positive mode, α-CHCA matrix): *m*/*z* 617.63 $[M + Na]^+$, calcd for $[C_{28}H_{46}N_2O_8Si_2Na]^+$ 617.27; elemental analysis: calcd (%) for $C_{28}H_{46}N_2O_8Si_2$: C, 56.54; H, 7.79; N, 4.71; found: C, 56.63; H, 7.65; N, 4.52.

General procedure for the peptide coupling. The amine (1 equiv), the carboxylic acid (1 equiv) and BOP phosphonium salt (1 equiv) were dissolved in dry CH_3CN (0.1 M). Then, DIEA (3 equiv) was added and the resulting reaction mixture was stirred at room temperature for 45 min. The solvent was removed under vacuum, the resulting oil was dissolved in CH_2Cl_2 and washed with a sat. solution of $NaHCO_3$. The aqueous layer was extracted with CH_2Cl_2 and the combined organic layers were washed with deionised water, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified over silica gel with CH_2Cl_2–AcOEt as eluents.

Boc-Asp(TMSE)-Glu(TMSE)-OBn **10** (yellow oil, 98%): R_f (CH_2Cl_2–AcOEt, 7 : 3, v/v) 0.5; $[\alpha]^{21}_{365}$ +44.7° (*c* 1.06 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.03 (s, 18H, 2Si(C*H*$_3$)$_3$), 0.93–1.01 (m, 4H, 2C*H*$_2$Si), 1.45 (s, 9H, C(C*H*$_3$)$_3$), 1.97–2.04 (m, 1H, 0.5C*H*$_{2Glu}$), 2.19–2.37 (m, 3H, C*H*$_{2Glu}$CO + 0.5C*H*$_{2Glu}$), 2.62 (dd, J = 6.0 and 17.0 Hz, 1H, 0.5C*H*$_{2Asp}$), 2.99 (dd, J = 5.0 and 17 Hz, 1H, 0.5C*H*$_{2Asp}$), 4.11–4.20 (m, 4H, 2OC*H*$_2$), 4.46–4.54 (m, 1H, C**H*$_{Asp}$), 4.59–4.66 (m, 1H, C**H*$_{Glu}$), 5.16 (s, 2H, ArC*H*$_2$ O), 5.68 (d, J = 8.0 Hz, 1H, N*H*$_{Asp}$), 7.18 (d, J = 8.0 Hz, 1H, N*H*$_{Glu}$), 7.34 (s, 5H, 5Ar*H*); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ –1.2 (6C), 17.6 (2C), 27.7, 28.6 (3C), 30.4, 36.5, 50.8, 52.1, 63.2, 63.7, 67.6, 80.8, 128.5–128.9 (3 peaks, 5C), 135.5, 155.7, 171.1, 171.6, 172.5, 173.1; IR (neat): ν_{max} 3350, 2955, 2899, 1732, 1682, 1520, 1455, 1391, 1367, 1250, 1170, 1062; MS (MALDI-TOF, positive mode, α-CHCA matrix): *m*/*z* 675.62 $[M + Na]^+$, calcd for $[C_{31}H_{52}N_2O_9Si_2Na]^+$ 675.31; elemental analysis: calcd (%) for $C_{31}H_{52}N_2O_9Si_2$: C, 57.03; H, 8.03; N, 4.29; found: C, 57.13; H, 7.71; N, 4.26.

Boc-Val-Asp(TMSE)-OBn **16** (colorless oil, quantitative yield): R_f (CH_2Cl_2–AcOEt, 96 : 4, v/v) 0.5; $[\alpha]^{21}_{365}$ +27.1° (*c* 0.99 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.02 (s, 9H, Si(C*H*$_3$)$_3$), 0.83–0.93 (m, 8H, C*H*$_2$Si + 2C*H*$_{3Val}$), 1.43 (s, 9H, C(C*H*$_3$)$_3$),

2.03–2.10 (m, 1H, C*H*CH_3), 2.78 (dd, J = 4.5 and 17.0 Hz, 1H, 0.5CH_2), 3.05 (dd, J = 4.5 and 17.0 Hz, 1H, 0.5CH_2), 3.94–3.98 (m, 1H, C*H_{Val}), 4.11 (t, J = 9.0 Hz, 2H, OCH_2), 4.86–4.90 (m, 1H, C*H_{Asp}), 5.09–5.21 (m, 3H, ArCH_2O + NH_{Val}), 6.82 (d, J = 8.0 Hz, 1H, NH_{Asp}), 7.29 (s, 5H, 5Ar*H*); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ –1.2 (3C), 17.6, 17.8, 19.4, 28.6 (3C), 31.7, 36.6, 48.7, 59.9, 63.9, 67.9, 80.1, 128.7–128.9 (2 peaks, 5C), 135.4, 156.0, 170.7, 171.5, 171.6; IR (neat): ν_{max} 3324, 2960, 2900, 1732, 1661, 1505, 1456, 1391, 1366, 1289, 1250, 1173, 1046; MS (MALDI-TOF, positive mode, α-CHCA matrix): m/z 545.63 [M + Na]$^+$, calcd for $[C_{26}H_{42}N_2O_7SiNa]^+$ 545.27; elemental analysis: calcd (%) for $C_{26}H_{42}N_2O_7Si$: C, 59.74; H, 8.10; N, 5.36; found: C, 59.24; H, 8.07; N, 5.31.

Ac-Asp(TMSE)-Glu(TMSE)-Val-Asp(TMSE)-OBn **18** (beige solid, 96%): R_f 0.5 (CH_2Cl_2–AcOEt, 1 : 1, v/v); $[\alpha]^{21}_{365}$ –36.4° (c 0.95 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.01 (s, 9H, Si(CH_3)$_3$), 0.02 (s, 9H, Si(CH_3)$_3$), 0.03 (s, 9H, Si(CH_3)$_3$), 0.82–1.01 (m, 12H, 3CH_2Si + 2CH_{3Val}), 2.05–2.22 (m, 6H, C*H*CH_3 + CH_{2Glu} + CH_3), 2.42–2.49 (m, 2H, CH_{2Glu}CO), 2.70–3.15 (m, 4H, 2CH_{2Asp}), 4.02–4.19 (m, 6H, 3OCH_2), 4.27–4.38 (m, 2H, C*H_{Val} + C*H_{Glu}), 4.72–4.76 (m, 1H, C*H_{Asp}), 4.87–4.95 (m, 1H, C*H_{Asp}), 5.11 (Abd, J = 12 Hz, 1H, 0.5ArCH_2O), 5.19 (Abd, J = 12 Hz, 1H, 0.5ArCH_2O), 6.91 (d, J = 8.0 Hz, 1H, NH_{Asp}), 7.07 (d, J = 8.0 Hz, 1H, NH_{Asp}), 7.12 (d, J = 8.0 Hz, 1H, NH_{Val}), 7.32 (s, 5H, 5Ar*H*), 7.79 (d, J = 8.0 Hz, 1H, NH_{Glu}); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ –1.2 (9C), 17.5–17.6 (2 peaks, 3C), 18.0, 19.5, 23.4, 26.6, 30.7, 31.1, 36.2, 36.6, 49.0, 49.9, 54.5, 58.9, 63.7, 63.9, 64.0, 67.9, 128.7–128.9 (3 peaks, 5C), 135.5, 170.9, 171.0, 171.1, 171.3, 171.4, 171.7, 172.5, 175.0; IR (KBr): ν_{max} 3276, 2955, 1737, 1634, 1544, 1454, 1385, 1250, 1167; MS (MALDI-TOF, positive mode, α-CHCA matrix): m/z 931.57 [M + Na]$^+$, calcd for $[C_{42}H_{72}N_4O_{12}Si_3Na]^+$ 931.44; elemental analysis: calcd (%) for $C_{42}H_{72}N_4O_{12}Si_3$, 0.5$CH_2Cl_2$: C, 53.63; H, 7.73; N, 5.89; found: C, 53.36; H, 7.40; N, 5.89.

Synthesis of PGA chemiluminescent probe (23). *(a) Coupling of enol ether*: Phenylacetamide **20** (656 mg, 1.91 mmol) was dissolved in dry DMF (10 mL). Phenolic enol ether **21** (520 mg, 1.92 mmol), TEA (800 µL, 5.76 mmol) and BOP phosphonium salt (950 mg, 2.15 mmol) were sequentially added. The resulting reaction mixture was stirred at room temperature for 6 h. The solvent was removed under vacuum, the resulting oily residue was dissolved in AcOEt and washed with deionised water. The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude product was purified by chromatography on silica gel using cyclohexane–AcOEt (4 : 6, v/v) as the mobile phase, yielding the corresponding aryl ester **22** as a white solid (606 mg, 53%). R_f (cyclohexane-AcOEt, 4 : 6, v/v) 0.45; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.55–1.90 (m, 12H), 2.66 (br s, 1H), 3.25 (br s, 1H), 3.30 (s, 3H), 3.53 (s, 2H), 3.81 (s, 3H), 3.85 (s, 2H), 3.88 (s, 3H), 4.44 (d, J = 5.3 Hz, 2H), 6.77 (s, 1H), 6.80 (s, 1H), 6.84 (ddd, J = 7.9 Hz, 2.3 Hz and 0.8 Hz, 1H), 6.91 (t, J = 2.3 Hz, 1H), 7.00-7.15 (m, 6H), 7.21 (t, J = 7.9 Hz, 1H); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 28.3, 30.3, 32.2, 37.2, 38.3, 39.1, 39.3, 41.4, 43.9, 56.0, 56.1, 58.0, 112.9, 113.5, 120.4, 122.1, 124.1, 127.1, 127.3, 128.9, 129.1, 129.4, 132.9, 134.9, 137.3, 142.6, 148.5, 148.6, 150.6; MS (MALDI-TOF, positive mode, α-CHCA matrix): m/z 618.30 [M + Na]$^+$, calcd for $[C_{37}H_{41}NO_6Na]^+$ 618.28; elemental analysis: calcd (%) for $C_{37}H_{41}NO_6$: C, 74.60; H, 6.94; N, 2.35; found: C, 74.25; H, 7.09; N, 2.41. *(b) Oxidation:* $P(OC_6H_5)_3$ (12.5 µL, 47.5 µmol, 2.7 equiv) was introduced into a 2-neck round bottom flask (10 mL) containing 2.5 mL of dry CH_2Cl_2. The solution was cooled to –78 °C and ozone (O_3) was bubbled until a blue color (excess of O_3) appeared. Then, the reaction medium was purged with argon gas until the solution became colorless (45 min). Enol ether **22** (10 mg, 16.8 µmol) in solution in dry CH_2Cl_2 (0.5 mL) was added and the resulting reaction mixture was stirred at –78 °C for 30 min, warmed to –30 °C and then stirred again for 1 h. The oxidation reaction was checked for completion by RP-HPLC (system A) and the mixture was evaporated to dryness. The resulting yellow oily residue was purified by semi-preparative RP-HPLC (system B). The product-containing fractions were lyophilised to give the 1,2-dioxetane **23** as a white powder (4.1 mg, 39%). A badly resolved ^{1}H NMR spectrum was obtained. MS (ESI+): m/z 650.07 [M + H]$^+$, calcd for $[C_{37}H_{41}NO_8]^+$ 650.27; HPLC (system A): t_R = 22.2 min, purity 95%.

Synthesis of PGA chemiluminescent probe (29). *(a) Coupling of enol ether*: The sodium salt of phenolic enol ether **21-Na** (0.15 mmol) was suspended in dry CH_3CN (1 mL). Crown ether 15-C-5 (5 µL, 0.025 mmol, 0.17 equiv) was added and the resulting solution was stirred for 5 min. Then, a solution of mesylate ester **27** (0.2 mmol) in a mixture of dry CH_3CN (1 mL) and dry DMF (500 µL) was added and the resulting reaction mixture was stirred at room temperature for 6 h. Thereafter, the mixture was concentrated under vacuum, AcOEt (15 mL) was added and the solution was washed with aq. Na_2CO_3 (10%, 5 mL) and deionised water (5 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated. The crude product was purified over a silica gel pad using a mixture of cyclohexane–AcOEt (85 : 15, v/v), affording **28** as a beige solid (40 mg, 54%). R_f (cyclohexane–AcOEt, 75 : 25, v/v) 0.5; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.73–1.98 (m, 12H, 2C$H_{Adam.}$ + 5C$H_{2Adam.}$), 2.61 (m, 1H, C$H_{Adam.}$), 3.23 (s, 1H, C$H_{Adam.}$), 3.27 (s, 3H, OCH_3), 3.75 (s, 2H, ArCH_2CO), 5.00 (s, 2H, ArCH_2O), 6.82–6.91 (m, 3H, 3Ar*H*), 7.04 (s, 1H, Ar*H*), 7.32-7-47 (m, 9H, 5Ar*H* + 4ArH_{PABA}); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 28.6 (2C), 30.5, 32.5, 37.5, 39.3 (2C), 39.5 (2C), 45.0, 58.0, 69.8, 114.3, 115.9, 120.2 (2C), 122.5, 128.0, 128.6 (2C), 129.2, 129.5 (2C), 129.8 (2C), 132.0, 133.3, 134.7, 137.2, 137.7, 143.6, 158.7, 169.5; IR (KBr): ν_{max} 3296, 2909, 2848, 2359, 1666, 1607, 1538, 1412, 1281, 1238, 1200, 1080, 1026; MS (ESI+): m/z 494.13 [M + H]$^+$, calcd for $[C_{33}H_{36}NO_3]^+$ 494.27. *(b) Oxidation:* $P(OC_6H_5)_3$ (56 µL, 0.21 mmol, 3.0 equiv) was introduced into a 2-neck round bottom flask (25 mL) containing 1.5 mL of dry CH_2Cl_2. The solution was cooled to –78 °C and ozone (O_3) was bubbled until a blue color (excess of O_3) appeared. Then, the reaction medium was purged with argon gas until the solution became colorless. Enol ether **28** (35 mg, 0.070 mmol) in solution in dry CH_2Cl_2 (2.5 mL) was added and the resulting reaction mixture was allowed to warm at room temperature. After 2 h, the formation of the 1,2-dioxetane was checked by TLC and showed the complete conversion of the starting material. The reaction mixture was concentrated under reduced pressure and the resulting solid was purified by chromatography on a silica gel column using a mixture of cyclohexane–AcOEt (7 : 3, v/v) as the mobile phase, affording **29** as a white solid (27 mg, 73%). R_f (cyclohexane–AcOEt, 7 : 3, v/v) 0.5; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.52–1.95 (m, 12H, 2C$H_{Adam.}$ + 5C$H_{2Adam.}$), 2.11 (m, 1H, C$H_{Adam.}$), 3.01 (s, 1H, C$H_{Adam.}$), 3.20 (s, 3H, OCH_3), 3.75 (s, 2H, ArCH_2CO), 5.05

(s, 2H, ArCH_2O), 6.95–7.08 (m, 2H, 2ArH), 7.22-7-46 (m, 11H, 7ArH + 4ArH_{PABA}); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 26.2, 26.3, 31.8, 31.9, 32.6, 33.2, 33.4, 35.0, 36.7, 45.0, 50.2, 69.9, 95.8, 112.3, 116.5, 120.1 (2C), 120.4, 126.0, 128.0, 128.5 (2C), 129.5 (2C), 129.8 (2C), 130.2, 133.0, 134.7, 136.4, 137.8, 158.5, 169.4; MS (ESI+): m/z 543.07 [M + H]$^+$, calcd for $[C_{33}H_{37}NO_6]^+$ 543.26.

Ac-Asp(TMSE)-Glu(TMSE)-Val-Asp(TMSE)-PABA-OH (30a). Side-chain protected tetrapeptide **19** (598 mg, 0.73 mmol) was dissolved in dry DMF (7 mL) and the resulting solution was cooled to 0 °C. Then, PABA (90 mg, 0.73 mmol, 1 equiv) and the HCl salt of EDC, (154 mg, 0.80 mmol, 1.1 equiv) were sequentially added and the resulting reaction mixture was stirred at room temperature for 1 h. The solvent was removed under vacuum, the resulting solid was dissolved in CH_2Cl_2 (40 mL), washed with aq. citric acid (10%, 15 mL) and deionised water (20 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by chromatography on a silica gel column using a mixture of CH_2Cl_2–AcOEt (3 : 7, v/v) as the mobile phase, yielding the peptide–PABA conjugate as a 9:1 mixture of the two diastereoisomers **30a** and **30b** which was resolved in the next step (yellow solid, 580 mg, 86%). For analytical purpose, the mixture was purified by semi-preparative RP-HPLC (system D) to get **30a** in a pure form. R_f (CH_2Cl_2–AcOEt, 3 : 7, v/v) 0.5; $[\alpha]^{21}_{365}$ −125.5° (c 1.1 in $CHCl_3$); ^{1}H NMR (300 MHz, CD_3OD): δ 0.07 (s, 27H, 3Si(CH_3)$_3$), 0.99–1.04 (m, 12H, 3CH_2Si + 2CH_{3Val}), 1.95–2.10 (m, 4H, 0.5CH_{2Glu} + CH_3), 2.10–2.18 (m, 2H, CHCH$_3$ + 0.5CH_{2Glu}), 2.41–2.48 (m, 2H, CH_{2Glu}CO), 2.7–3.1 (m, 4H, 2CH_{2Asp}), 4.05–4.25 (m, 7H, C*H_{Val} + 3OCH_2), 4.36–4.42 (m, 1H, C*H_{Glu}), 4.58 (s, 2H, ArCH_2O), 4.70–4.76 (m, 1H, C*H_{Asp}), 4.80–4.95 (m, 1H, C*H_{Asp}), 7.33 (d, J = 8.0 Hz, 2H, 2ArH), 7.63 (d, J = 8.0 Hz, 2H, 2ArH), 7.94 (d, J = 7.0 Hz, 1H, NH_{Val}), 8.31 (d, J = 8.0 Hz, 1H, NH_{Asp}), 8.42–8.47 (m, 2H, NH_{Glu} + NH_{Asp}), 9.48 (s, 1H, OH); ^{13}C NMR (75.4 MHz, CD_3OD): δ −0.6 (9C), 19.1 (3C), 20.0, 20.5, 23.4, 28.4, 32.1, 32.3, 37.7, 37.9, 52.3, 53.1, 55.7, 62.2, 64.8, 65.1, 65.3, 65.7, 122.2 (2C), 129.4 (2C), 139.3, 139.7, 171.4, 173.0, 173.4, 174.2(6), 174.3(4), 174.4, 175.2, 175.7; IR (KBr): ν_{max} 3286, 2957, 1738, 1634, 1539, 1251, 1172; MS (ESI+): m/z 946.40 [M + Na]$^+$, calcd for $[C_{42}H_{73}N_5O_{12}Si_3Na]^+$ 946.45.

4-Nitrophenyl carbonate derivative (31). *para*-Nitrophenyl chloroformate (87 mg, 0.44 mmol, 4 equiv) and dry pyridine (44 μL, 0.54 mmol, 5 equiv) were mixed in dry CH_2Cl_2 and a white precipitate was formed. Alcohol **30a-b** (100 mg, 0.11 mmol) was added and the reaction mixture turned slowly to yellow and became homogenous. After 16 h of stirring at room temperature, CH_2Cl_2 was added and the solution was washed with aq. citric acid (10%) and deionised water. The organic phase was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting solid was purified by chromatography on a silica gel column using a mixture of CH_2Cl_2–AcOEt (6 : 4, v/v) as the mobile phase, affording activated carbonate **31** as a yellow solid (86 mg, 72%). R_f (CH_2Cl_2–AcOEt, 6 : 4, v/v) 0.5; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.02 (s, 9H, Si(CH_3)$_3$), 0.03 (s, 9H, Si(CH_3)$_3$), 0.05 (s, 9H, Si(CH_3)$_3$), 0.80–1.05 (m, 12H, 3CH_2Si + 2CH_{3Val}), 2.02–2.20 (m, 5H, CH_{2Glu} + CH_3), 2.28–2.42 (m, 1H, CHCH$_3$), 2.45–2.70 (m, 2H, CH_{2Glu}CO), 2.82–3.14 (m, 4H, 2CH_{2Asp}), 4.02–4.28 (m, 8H, C*H_{Val} + C*H_{Glu} + 3OCH_2), 4.66–4.74 (m, 1H, C*H_{Asp}), 4.92–5.08 (m, 1H, C*H_{Asp}), 5.23 (s, 2H, ArCH_2O), 7.09 (d, J = 7.2 Hz, 1H, NH_{Val}), 7.21 (d, J = 6.8 Hz, 1H, NH_{Asp}), 7.32–7.40 (m, 4H, 4ArH), 7.59 (d, J = 8.7 Hz, 1H, NH_{Asp}), 7.79 (d, J = 8.3 Hz, 2H, 2ArH), 8.27 (d, J = 8.3 Hz, 2H, 2ArH), 8.50 (d, J = 7.5 Hz, 1H, NH_{Glu}), 8.71 (s, 1H, ArNHCO); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ −1.23 (9C), 17.6 (3C), 19.5 (2C), 23.3, 25.4, 29.4, 30.0, 31.5, 36.0, 36.8, 50.2, 51.0, 56.8, 60.6, 63.6, 64.0, 64.3, 71.1, 120.5, 122.2, 125.6, 129.9, 139.3, 145.6, 152.7, 155.9, 169.1, 171.3, 171.4, 171.8, 172.0, 172.3, 172.7, 172.9, 175.9; HPLC (system C) : t_R = 34.5 min, purity >90%.

Enol ether (32). Phenolic enol ether **21** (19 mg, 0.070 mmol, 1.5 equiv) and anhydrous K_2CO_3 (13 mg, 0.094 mmol, 2 equiv) were mixed in dry CH_3CN (400 μL) and the reaction mixture was stirred at room temperature for 20 min. Then, activated carbonate **28** (51 mg, 0.047 mmol) was added and the reaction mixture was stirred at room temperature for 16 h. The solution was concentrated and CH_2Cl_2 (10 mL) was added. The organic phase was washed with deionised water (5 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting solid was purified by chromatography on a silica gel column using a mixture of CH_2Cl_2–AcOEt (1 : 1, v/v) as the mobile phase, affording **32** as a white solid (33 mg, 58%). R_f (CH_2Cl_2–AcOEt, 1 : 1, v/v) 0.5; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.02 (s, 27H, 3Si(CH_3)$_3$), 0.90–1.05 (m, 12H, 3CH_2Si + 2CH_{3Val}), 1.55–2.00 (m, 17H, 5C$H_{2Adam.}$ + 2C$H_{Adam.}$ + CH_3 + CH_{2Glu}), 2.20–2.45 (m, 1H, CHCH$_3$), 2.45–2.70 (m, 3H, C$H_{Adam.}$ + CH_{2Glu}CO), 2.80–3.02 (m, 4H, 2CH_{2Asp}), 3.22 (s, 1H, C$H_{Adam.}$), 3.28 (s, 3H, OCH_3), 4.05–4.25 (m, 8H, C*H_{Val} + C*H_{Glu} + 3OCH_2), 4.68–4.82 (m, 1H, C*H_{Asp}), 4.92–5.05 (m, 1H, C*H_{Asp}), 5.20 (s, 2H, ArCH_2O), 7.05–7.38 (m, 6H, 4ArH + 2ArH_{PABA}), 7.70 (d, J = 8.7 Hz, 2H, 2ArH_{PABA}), 8.65 (s, 1H, ArNHCO); HPLC (system E) : t_R = 27.8 min, purity >90%.

1,2-Dioxetane (33). $P(OC_6H_5)_3$ (20 μL, 0.076 mmol, 2.7 equiv) was introduced into a 2-neck round bottom flask (25 mL) containing 2 mL of dry CH_2Cl_2. The solution was cooled to −78 °C and O_3 was bubbled until a blue color (excess of O_3) appeared. Then, the reaction mixture was purged with argon gas until the solution became colorless. Enol ether **32** (34 mg, 0.028 mmol) in solution in dry CH_2Cl_2 (2 mL) was added and the resulting reaction mixture was allowed to warm at room temperature. After 1 h, the formation of the desired 1,2-dioxetane was checked by RP-HPLC (system E): the RP-HPLC elution profile showed *ca.* 50% conversion of the starting enol ether. The reaction mixture was concentrated under reduced pressure and the resulting solid was purified by semi-preparative RP-HPLC (system F) to give after lyophilisation the corresponding 1,2-dioxetane **33** as a white solid (7.0 mg, 20%). MS (ESI+): m/z 1156.27 [M − TMSEOH + Na]$^+$, calcd for $[C_{56}H_{79}N_5NaO_{16}Si_2]^+$ 1156.50, under ionisation conditions, the release of one unit of TMSE with subsequent formation of an aspartimide was observed. HPLC (system E) : t_R = 26.3 min, purity >90%.

Synthesis of caspase-3 chemiluminescent probe (37). *(a) Coupling of enol ether*: Phenolic enol ether **21** (46 mg, 0.17 mmol, 1 equiv) was dissolved in dry HPLC-grade acetone (1 mL). Anhydrous K_2CO_3 (47 mg, 0.34 mmol, 2 equiv) was added and the resulting mixture was stirred for 20 min. Then, mesylate ester **34** in solution in dry HPLC-grade acetone (1.5 mL) was added and the mixture was stirred at room temperature for 16 h. The reaction was checked to completion by TLC and the reaction mixture

was diluted with CH_2Cl_2 (15 mL) and washed with deionised water (7 mL). The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified by chromatography on a silica gel column using a mixture of CH_2Cl_2–AcOEt (1 : 1, v/v) as the mobile phase, affording **35** as a pale yellow oil (88 mg, 44% over 2 steps from **27**). R_f (CH_2Cl_2–AcOEt, 1 : 1, v/v) 0.5; $[\alpha]^{21}_{365}$ −23.7° (*c* 0.95 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.02 (s, 9H, Si(CH_3)$_3$), 0.04 (s, 9H, Si(CH_3)$_3$), 0.06 (s, 9H, Si(CH_3)$_3$), 0.92–0.98 (m, 12H, 3CH_2Si + 2CH_{3Val}), 1.77–1.94 (m, 15H, 2C$H_{Adam.}$ + 5C$H_{2Adam.}$ + CH_3), 2.31–2.42 (m, 3H, CHCH$_3$ + CH_{2Glu}), 2.50–2.63 (m, 3H, C$H_{Adam.}$ + CH_{2Glu}CO), 2.83–2.95 (m, 4H, 2CH_{2Asp}), 3.22 (s, 1H, C$H_{Adam.}$), 3.27 (s, 3H, OCH_3), 3.84–3.89 (m, 1H, C*H_{Asp}), 4.05–4.20 (m, 7H, C*H_{Val} + 3OCH_2), 4.69–4.75 (m, 1H, C*H_{Glu}), 4.98 (s$_{br}$, 3H, ArCH_2O + C*H_{Asp}), 6.82–6.92 (m, 3H, 3ArH), 7.14 (d, J = 8.0 Hz, 1H, NH_{Glu}), 7.19–7.25 (m, 2H, NH_{Asp} + ArH), 7.37 (d, J = 9.0 Hz, 4H, 2ArH_{PABA} + NH_{Asp} + NH_{Val}), 7.68 (s, 2H, 2ArH_{PABA}), 8.62 (s, 1H, ArNH); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ −1.2 (9C), 17.6 (3C), 18.3, 19.5, 23.3, 26.7, 28.6 (2C), 29.8, 30.0, 30.5, 31.4, 32.5, 35.6, 36.0, 37.5, 39.4 (2C), 39.5 (2C), 49.8, 50.5, 54.1, 58.2, 63.4 (2C), 63.8, 70.0, 114.4, 115.8, 120.3 (2C), 122.5, 128.7 (2C), 129.2, 132.2, 133.1, 137.2, 138.1, 138.2, 143.6, 168.8, 170.2, 171.3, 171.7, 172.0, 173.3, 174.5, 175.8; IR (KBr): ν_{max} 3322, 2925, 1720, 1655, 1536; MS (ESI+): *m*/*z* 1198.53 [M + Na]$^+$, calcd for [$C_{60}H_{93}N_5O_{13}Si_3Na$]$^+$ 1198.60; HPLC (system C) : t_R = 39.6 min, purity >98%. *(b) Oxidation*: P(OC_6H_5)$_3$ (22 μL, 0.085 mmol, 2.7 equiv) was introduced into a 2-neck round bottom flask (25 mL) containing 2 mL of dry CH_2Cl_2. The resulting solution was cooled to −78 °C and O_3 was bubbled until a blue color (excess of O_3) appeared. Then, the reaction mixture was purged with argon gas until the solution became colorless. Enol ether **35** (37 mg, 0.031 mmol) in solution in dry CH_2Cl_2 (2 mL) was added and the resulting reaction mixture was allowed to warm at room temperature. After 1 h, the formation of the 1,2-dioxetane was checked by RP-HPLC (system E): the RP-HPLC elution profile showed *ca.* 50% conversion of the starting material. The reaction mixture was concentrated under reduced pressure and the resulting solid was purified by semi-preparative RP-HPLC (system F), to give after lyophilisation the targeted 1,2-dioxetane **36** as a white amorphous powder (10 mg, 25%, 50% bsrm). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.03 (s, 27H, 3Si(CH_3)$_3$), 0.94–1.02 (m, 12H, 3CH_2Si + 2CH_{3Val}), 1.43–1.78 (m, 12H, 2C$H_{Adam.}$ + 5C$H_{2Adam.}$), 1.98 (s, 3H, CH_3), 2.22–2.52 (m, 6H, CHCH$_3$ + C$H_{Adam.}$ + CH_2CO$_{Glu}$ + CH_{2Glu}), 2.78–3.02 (m, 4H, 2CH_{2Asp}), 3.20 (s, 1H, C$H_{Adam.}$), 3.21 (s, 3H, OCH_3), 3.80–3.92 (m, 1H, C*H_{Asp}), 4.08–4.22 (m, 7H, C*H_{Val} + 3OCH_2), 4.75–4.82 (m, 1H, C*H_{Glu}), 5.05 (s$_{br}$, 3H, ArCH_2O + C*H_{Asp}), 6.36–6.40 (m, 1H, ArH), 6.99 (d, J = 6.0 Hz, 1H, ArH), 7.30–7.38 (m, 4H, 2ArH_{PABA} + 2ArH), 7.71 (d, J = 8.0 Hz, 2H, 2ArH_{PABA}), 8.54–8.60 (m, 1H, NHAc); IR (KBr): ν_{max} 3310, 2917, 1719, 1655, 1542, 1389, 1250, 1165; MS (ESI+): *m*/*z* 1309.53 [M + H]$^+$, calcd for [$C_{66}H_{109}N_6O_{15}Si_3$]$^+$ 1309.73; HPLC (system C) : t_R = 37.8 min, purity >98%. *(c) Removal of TMSE protecting groups*: The latter fully-protected 1,2-dioxetane **36** (2.0 mg, 1.53 μmol) was dissolved in dry DMSO (200 μL). TBAF (1.0 M in THF, 31 μL, 31 μmol, 20 equiv) was added to the solution, allowing the quantitative removal of the TMSE groups (followed by RP-HPLC analysis after 5 min of reaction, system C). The mixture was diluted with TEAB buffer (50 mM, pH 7.5) and purified by semi-preparative RP-HPLC (system G). The product-containing fractions were lyophilised to give the target caspase-3 chemimuminescent probe **37** as a colorless oil. A stock solution of this chemiluminescent probe was prepared in DMSO and quantification was achieved by UV–visible absorption measurements in deionised water at λ_{max} = 250 nm (quantitative yield after RP-HPLC purification). HPLC (system C): t_R = 27.1 min, purity >90%; UV–vis (deionised water, 25 °C): λ_{max} (ε) = 250 nm (19 000 L mol^{-1} cm^{-1}); MS (ESI-): *m*/*z* 906.33 [M − H]$^-$, calcd for [$C_{45}H_{56}N_5O_{15}$]$^-$ 906.38.

General procedure for the *in vitro* cleavage of chemiluminescent probes (23), (29) and (37) by recombinant proteases

Enzymatic assay with recombinant human caspase-3: 1,2-Dioxetane **37** was dissolved in caspase-3 buffer (200 μL, 10% (w/v) sucrose, 50 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% (w/v) CHAPS, pH 7.4, final concentration 75 μM). 1 μL of recombinant human caspase-3 (1.6 10^{-3} U) was added and the enzymatic reaction mixture was incubated at 37 °C for an overall time of 120 min. Every 15 min, an aliquot (20 μL) was sampled and added to an alkaline caspase-3 buffer (30 μL, same caspase-3 buffer at pH 12.3, final concentration: 30 μM) containing the enhancers (CTAB, 2 mM, 5-(stearoylamino)fluorescein 0.18 mM). Luminescence spectra were recorded (into an ultra-micro fluorescence cell Hellma®, 105.51-QS, 10 × 3 mm, 45 μL) for each aliquot between 490 and 570 nm for 36 min under cycle scan mode (240 scans, scan time duration = 9 s) with the following parameters: bio/chemiluminescence mode with gate time = 100 ms, emission filter: open and PMT detector voltage level: high.

Caspase-3 detection limit: To determine the detection limit of the self-cleavable chemiluminescent probe **37**, a decreasing amount of caspase-3 enzyme was incubated during 60 min with a solution of **37** (30 μM). The total light emission was recorded between 490 and 570 nm during 10 min and reported as a function of the enzyme concentration. A detection limit of around 1.0 pmol was observed.

Representative control experiments with other proteases: To demonstrate the specificity of the cleavage by caspase-3 enzyme, the self-cleavable chemiluminescent probe **37** was incubated with human recombinant caspase-9 (1 μL, 2.38 U in a caspase-9 buffer: 0.1 M MES, 10% PEG, 0.1% CHAPS, 10 mM DTT, pH 6.5) and *A. faecalis* recombinant PGA (1.2 mg, 1.9 U in 50 mM phosphate buffer, pH 7.5) for 60 min. Then, the enzymatic reaction mixture was added onto the alkaline caspase-3 buffer (pH 12.3, final concentration of **34**: 30 μM) containing the enhancers (CTAB, 2 mM, 5-(stearoylamino)fluorescein 0.18 mM) and light emission was recorded under the same conditions than described above. No light could be detected for both enzymes.

Enzymatic assay with PGA: 1,2-Dioxetane **23** or **29** was dissolved in phosphate buffer (50 mM, pH 7.5, final concentration: 75 μM). 10 μL of a solution of recombinant PGA in PBS (5.0 mg in 3.0 mL of phosphate buffer, 1.05 10^{-2} U) were added and the enzymatic reaction mixture was incubated at 37 °C for an overall time of 120 min. Every 5 min, an aliquot (20 μL) was sampled and added to the same alkaline buffer (30 μL, final concentration: 30 μM) used for **37**. Luminescence spectra were recorded (into an ultra-micro fluorescence cell Hellma®, 105.51-QS, 10 × 3 mm, 45 μL) for each aliquot under the same conditions as described for caspase-3 chemiluminescent probe.

Abbreviations

AcOEt	ethyl acetate
Asp	aspartic acid
Boc	*tert*-butyloxycarbonyl
Bn	benzyl
BOP	benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHCA	α-cyano-4-hydroxycinnamic acid
CIEEL	chemically initiated electron exchange luminescence
CTAB	cetyltrimethylammonium bromide
DCC	*N*,*N*′-dicyclohexylcarbodiimide
DCU	*N*,*N*′-dicyclohexylurea
DIEA	*N*,*N*-diisopropylethylamine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDC	*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide
EDTA	ethylenediaminetetraacetic acid
EEDQ	2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline
FRET	fluorescence resonance energy transfer
Glu	glutamic acid
HATU	*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate
HOBt	1-hydroxybenzotriazole
MES	2-(*N*-morpholino)ethanesulfonic acid
PABA	*para*-aminobenzylic alcohol
PEG	poly(ethylene glycol)
PGA	penicillin G acylase
PIPES	1,4-piperazinediethanesulfonic acid
PTSA	*para*-toluenesulfonic acid
RP-HPLC	reversed-phase high pressure liquid chromatography
TAS-F	tris(dimethylamino)sulfonium difluorotrimethylsilicate
TBAF	tetrabutylammonium fluoride
TBP	2,4,6-tri-*tert*-butyl-pyridine
TEA	triethylamine
TEAA	triethylammonium acetate
TEAB	triethylammonium bicarbonate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TMSE	2-(trimethylsilyl)ethyl
TMSEOH	2-(trimethylsilyl)ethanol
TPP	tetraphenylporphyrin
Val	valine

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References

1 International-human-genome-sequencing-consortium, *Nature*, 2001, **409**, 860.
2 X. S. Puente, L. M. Sanchez, C. M. Overall and C. Lopez-Otin, *Nat. Rev. Genet.*, 2003, **4**, 544.
3 For recent reviews, see: (*a*) X. Chen, M. Sun and H. Ma, *Curr. Org. Chem.*, 2006, **10**, 477; (*b*) G. Blum, *Curr. Opin. Drug Discov. Devel.*, 2008, **11**, 708; (*c*) S. Lee, K. Park, K. Kim, K. Choi and I. C. Kwon, *Chem. Commun.*, 2008, 4250.
4 (*a*) A. Roda, P. Pasini, M. Guardigli, M. Baraldini, M. Musiani and M. Mirasoli, *Fresenius J. Anal. Chem.*, 2000, 752; (*b*) A. Roda, P. Pasini, M. Mirasoli, E. Michelini and M. Guardigli, *Trends Biotechnol.*, 2004, **22**, 295.
5 O. Shimomura, *Bioluminescence: Chemical Principles and Methods*, World Scientific, Singapore, 2006.
6 For recent examples, see: (*a*) Y. Zhang, M.-k. So, A. M. Loening, H. Yao, S. S. Gambhir and J. Rao, *Angew. Chem., Int. Ed.*, 2006, **45**, 4936; (*b*) A. Kanno, Y. Yamanaka, H. Hirano, Y. Umezawa and T. Ozawa, *Angew. Chem., Int. Ed.*, 2007, **46**, 7595; (*c*) H. Yao, M. K. So and J. Rao, *Angew. Chem., Int. Ed.*, 2007, **46**, 7031; (*d*) H. Yao, Y. Zhang, F. Xiao, Z. Xia and J. Rao, *Angew. Chem., Int. Ed.*, 2007, **46**, 4346.
7 M. Matsumoto, *J. Photochem. Photobiol. C*, 2004, **5**, 27.
8 K. Teranishi, *Bioorg. Chem.*, 2007, **35**, 82.
9 S. Beck and H. Koster, *Anal. Chem.*, 1990, **62**, 2258.
10 M. Matsumoto and N. Watanabe, *Bull. Chem. Soc. Jpn.*, 2005, **78**, 1899.
11 G. B. Schuster, *Acc. Chem. Res.*, 1979, **12**, 366.
12 W. Adam, D. Reinhardt and C. R. Saha-Möller, *Analyst*, 1996, **121**, 1527.
13 (*a*) I. Bronstein, J. C. Voyta, G. H. G. Thorpe, L. J. Krlcka and G. Armstrong, *Clin.Chem.*, 1989, **35**, 1441; (*b*) G. H. G. Thorpe, I. Bronstein, L. J. Kricka, B. Edwards and J. C. Voyta, *Clin. Chem.*, 1989, **35**, 2319.
14 R. C. Buxton, B. Edwards, R. R. Juo, J. C. Voyta, M. Tisdale and R. C. Bethell, *Anal. Biochem.*, 2000, **280**, 291.
15 S. Sabelle, P.-Y. Renard, K. Pecorella, S. de Suzzoni-Dezard, C. Creminon, J. Grassi and C. Mioskowski, *J. Am. Chem. Soc.*, 2002, **124**, 4874.
16 J. D. Stevenson, A. Dietel and N. R. Thomas, *Chem. Commun.*, 1999, 2105.
17 (*a*) *WO Pat.*, 9624849, 1996; (*b*) N. Watanabe, M. Ichikawa, A. Ono, H. Murakami and M. Matsumoto, *Chem. Lett.*, 2005, **34**, 718.
18 For recent applications of this self-immolative strategy to the enzyme-initiated release of phenol-based fluorophores, see: (*a*) Y. Meyer, J.-A. Richard, M. Massonneau, P.-Y. Renard and A. Romieu, *Org. Lett.*, 2008, **10**, 1517; (*b*) J.-A. Richard, M. Massonneau, P.-Y. Renard and A. Romieu, *Org. Lett.*, 2008, **10**, 4175 and reference 19.
19 J.-A. Richard, Y. Meyer, V. Jolivel, M. Massonneau, R. Dumeunier, D. Vaudry, H. Vaudry, P.-Y. Renard and A. Romieu, *Bioconjugate Chem.*, 2008, **19**, 1707.
20 *French Pat.*, 2886292, 2006.
21 W. Zhou, C. Andrews, J. Liu, J. W. Shultz, M. P. Valley, J. J. Cali, E. M. Hawkins, D. H. Klaubert, R. F. Bulleit and K. V. Wood, *ChemBioChem*, 2008, **9**, 714.
22 (*a*) P. A. Wender, E. A. Goun, L. R. Jones, T. H. Pillow, J. B. Rothbard, R. Shinde and C. H. Contag, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 10340; (*b*) L. R. Jones, E. A. Goun, R. Shinde, J. B. Rothbard, C. H. Contag and P. A. Wender, *J. Am. Chem. Soc.*, 2006, **128**, 6526.
23 J. A. Richard, L. Jean, A. Romieu, M. Massonneau, P. Noack-Fraissignes and P. Y. Renard, *Org. Lett.*, 2007, **9**, 4853.
24 S. Kothakota, T. Azuma, C. Reinhard, A. Klippel, J. Tang, K. Chu, T. J. McGarry, M. W. Kirschner, K. Koths, D. J. Kwiatkowski and L. T. Williams, *Science*, 1997, **278**, 294.
25 For recent examples of caspase-3 sensitive fluorogenic probes, see: (*a*) K. Bullok and D. Piwnica-Worms, *J. Med. Chem.*, 2005, **48**, 5404; (*b*) Z.-Q. Wang, J. Liao and Z. Diwu, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 2335; (*c*) K. E. Bullok, D. Maxwell, A. H. Kesarwala, S. Gammon,

J. L. Prior, M. Snow, S. Stanley and D. Piwnica-Worms, *Biochemistry*, 2007, **46**, 4055.
26 G. B. Fields and R. L. Noble, *Int. J. Pept. Protein Res.*, 1990, **35**, 161.
27 (*a*) M. Mergler, F. Dick, B. Sax, P. Weiler and T. Vorherr, *J. Peptide Sci.*, 2003, **9**, 36–46; (*b*) M. Mergler, F. Dick, B. Sax, C. Staehelin and T. Vorherr, *J. Peptide Sci.*, 2003, **9**, 518–526.
28 (*a*) H. Gerlach, *Helv. Chim. Acta*, 1977, **60**, 3039; (*b*) P. Sieber, *Helv. Chim. Acta*, 1977, **60**, 2711.
29 T. W. Greene and P. G. M. Wuts, *Protective Groups in Organic Synthesis*, 3rd edn., Wiley, New York, 1999.
30 R. K. Olsen, K. Ramasamy and T. Emery, *J. Org. Chem.*, 1984, **49**, 3527.
31 (*a*) B. Castro, J. R. Dormoy, B. Dourtoglou, G. Evin, C. Selve and J. C. Ziegler, *Synthesis*, 1976, 751; (*b*) B. Castro, J. R. Dormoy, G. Evin and C. Selve, *Tetrahedron Lett.*, 1975, **16**, 1219.
32 F. Kratz, I. A. Muller, C. Ryppa and A. Warnecke, *ChemMedChem*, 2008, **3**, 20.
33 (*a*) U. Grether and H. Waldmann, *Angew. Chem., Int. Ed.*, 2000, **39**, 1629; (*b*) U. Grether and H. Waldmann, *Chem.–Eur. J.*, 2001, **7**, 959.
34 B. Castro, G. Evin, C. Selve and R. Seyer, *Synthesis*, 1977, 413.
35 L. M. Stephenson and M. B. Zielinski, *J. Am. Chem. Soc.*, 1982, **104**, 5819.
36 (*a*) A. Greer, *Acc. Chem. Res.*, 2006, **39**, 797; (*b*) J.-A. Richard, *Synlett*, 2009, 1187.
37 J.-P. Ferezou, M. Julia, Y. Li, L. W. Liu and A. Pancrazi, *Bull. Soc. Chim. Fr.*, 1995, **132**, 428.
38 For the use of TAS-F in challenging selective deprotections of TMSE esters, see: (*a*) K. A. Scheidt, H. Chen, B. C. Follows, S. R. Chemler, D. S. Coffey and W. R. Roush, *J. Org. Chem.*, 1998, **63**, 6436; (*b*) K. A. Scheidt, T. D. Bannister, A. Tasaka, M. D. Wendt, B. M. Savall, G. J. Fegley and W. R. Roush, *J. Am. Chem. Soc.*, 2002, **124**, 6981; (*c*) A. El-Dahshan, S. Weik and J. Rademann, *Org. Lett.*, 2007, **9**, 949; (*d*) A. Fürstner and T. Nagano, *J. Am. Chem. Soc.*, 2007, **129**, 1906.
39 R. Erez and D. Shabat, *Org. Biomol. Chem.*, 2008, **6**, 2669.
40 N. Sewald and H.-D. Jakubke, *Peptides: Chemistry and Biology*, Wiley, Weinheim, 2002, pp. 205–209.
41 P. Reszka, K. Methling, M. Lalk, Z. Xiao, K. Weisz and P. J. Bednarski, *Tetrahedron: Asymmetry*, 2008, **19**, 49.
42 For a comprehensive review on peptide coupling reagents, see: E. Valeur and M. Bradley, *Chem. Soc. Rev.*, 2009, **38**, 606.
43 S. C. Jeffrey, M. Y. Torgov, J. B. Andreyka, L. Boddington, C. G. Cerveny, W. A. Denny, K. A. Gordon, D. Gustin, J. Haugen, T. Kline, M. T. Nguyen and P. D. Senter, *J. Med. Chem.*, 2005, **48**, 1344.
44 (*a*) S. Sabelle, J. Hydrio, E. Leclerc, C. Mioskowski and P.-Y. Renard, *Tetrahedron Lett.*, 2002, **43**, 3645; (*b*) E. L. Bastos, L. F. M. L. Ciscato, D. Weiss, R. Beckert and W. J. Baader, *Synthesis*, 2006, 1781.
45 (*a*) A. A. G. Shaikh and S. Sivaram, *Chem. Rev.*, 1996, **96**, 951; (*b*) J. P. Parrish, R. N. Salvatore and K. W. Jung, *Tetrahedron*, 2000, **56**, 8207.
46 (*a*) A. P. Schaap, H. Akhavan and L. J. Romano, *Clin. Chem.*, 1989, **35**, 1863; (*b*) *European Pat.*, 0352713, 1990; (*c*) W. Adam, I. Bronstein, B. Edwards, T. Engel, D. Reinhardt, F. W. Schneider, A. V. Trofimov and R. F. Vasil'ev, *J. Am. Chem. Soc.*, 1996, **118**, 10400.
47 H. E. Gottlieb, V. Kotlyar and A. Nudelman, *J. Org. Chem.*, 1997, **62**, 7512.