

Universal Access to Protease Chemiluminescent Probes through Solid-Phase Synthesis

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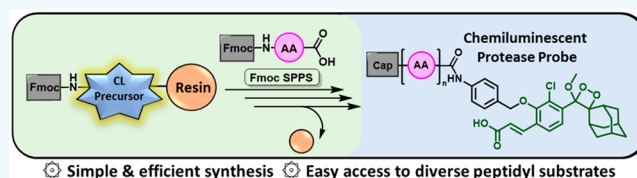
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Supporting Information

ABSTRACT: Protease chemiluminescent probes exhibit extremely high detection sensitivity for monitoring activity of various proteolytic enzymes. However, their synthesis, performed in solution, involves multiple synthetic and purification steps, thereby generating a major limitation for rapid preparation of such probes with diverse substrate scope. To overcome this limitation, we developed a general solid-phase-synthetic approach to prepare chemiluminescent protease probes, by peptide elongation, performed on an immobilized chemiluminescent enol-ether precursor.

The enol-ether precursor is immobilized on a 2-chlorotrityl-chloride resin through an acrylic acid substituent by an acid-labile ester linkage. Next, a stepwise elongation of the peptide is performed using standard Fmoc solid-phase peptide synthesis. After cleavage of the peptide-enol-ether precursor from the resin, by hexafluoro-iso-propanol, a simple oxidation of the enol-ether yields the final chemiluminescent dioxetane protease probe. To validate the applicability of the methodology, two chemiluminescent probes were efficiently prepared by solid-phase synthesis with dipeptidyl substrates designed for activation by aminopeptidase and cathepsin-B proteases. A more complex example was demonstrated by the synthesis of a chemiluminescent probe for detection of PSA, which includes a peptidyl substrate of six amino acids. We anticipate that the described methodology would be useful for rapid preparation of chemiluminescent protease probes with vast and diverse peptidyl substrates.



Proteases are a class of enzymes that are involved in almost every biological signaling and regulation processes in living systems.^{1,2} The ability of these enzymes, to cleave peptide bonds, is crucial for protein turnover in cells.³ In addition, proteases are involved not only in protein degradation but also in protein activation. Therefore, proteases are strongly associated with growth, cell division, differentiation, migration, and signaling. The classification of proteases is usually determined by their proteolytic mechanism, which involves various amino acid residues and includes cysteine-proteases, serine-proteases, threonine-proteases, and metalloproteases. Although these proteases mechanistically differ from one another, they all share one key principle: the ability to hydrolyze a specific amide bond in their peptidyl substrate. This amide bond breakdown is the central feature that enables scientists to design chemical tools for selective monitoring of protease activity.⁴

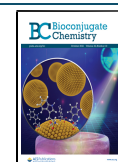
The most common method for monitoring protease activity is based on optical substrates, where fluorescence is the prominent modality.^{5,6} The general design and synthesis of turn-on fluorescent probes are presented in Figure 1A. Upon proteolytic cleavage of an amide bond located between a specific peptide and a fluorescent dye, an increase of a fluorescent signal, correlating to the catalytic activity of the protease, is produced. Such probes have been widely used to determine substrate specificity of proteases. In addition, they also provided valuable insights in regard to biological functions

of proteases and thus led to the discovery of new inhibitors and drugs.

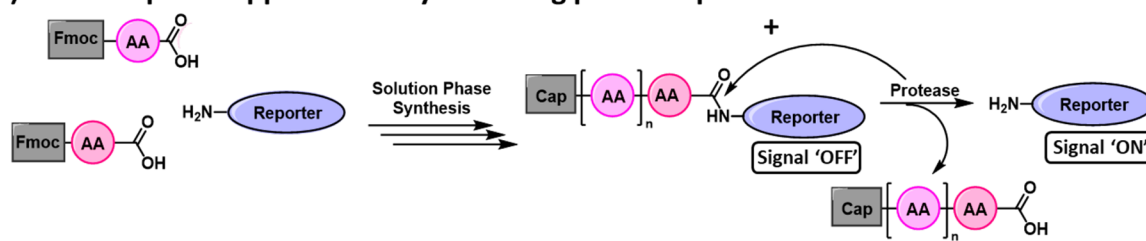
The synthesis of fluorogenic protease probes is usually performed in solution, where the C-terminus of a premade peptidyl substrate is coupled with an amino group of the fluorogenic dye (Figure 1A).^{7,8} This synthetic strategy consumes much time and resources, and requires challenging purification steps, especially when the peptidyl substrate involves the use of protecting groups.⁹ In addition, the synthesis in solution cannot be automated or be applied to efficiently prepare combinatorial substrate libraries for fluorescent probes.^{3,10} To overcome this limitation, researchers have developed a solid-phase peptide synthesis (SPPS) on a resin with an anchored fluorescent dye. The SPPS, introduced by Merrifield in the 1960s, is a fast and robust technique to synthesize peptides.^{11,12} Unlike synthesis in solution, SPPS can be automated and therefore can be used to create vast libraries of peptidyl substrates, in a method known as Positional Scanning Synthetic Combinatorial Libraries (PS-SCLs).^{13,14}

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A) Solution-phase approach for synthesizing protease probe



B) Fmoc Solid-Phase approach for synthesizing fluorescent protease probe

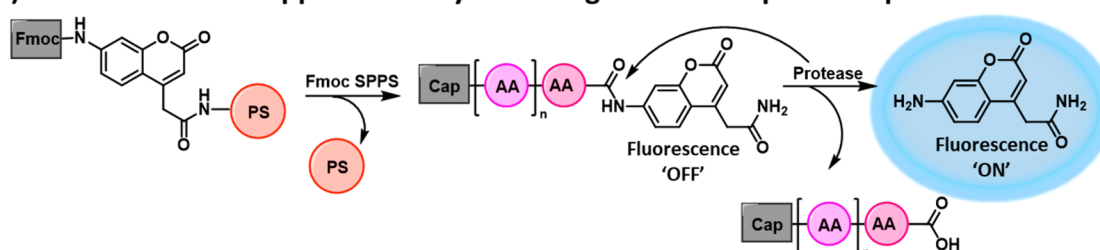


Figure 1. (A) Solution-phase synthesis approach to fluorescent protease probes. (B) Solid-phase synthesis approach to fluorescent protease probes.

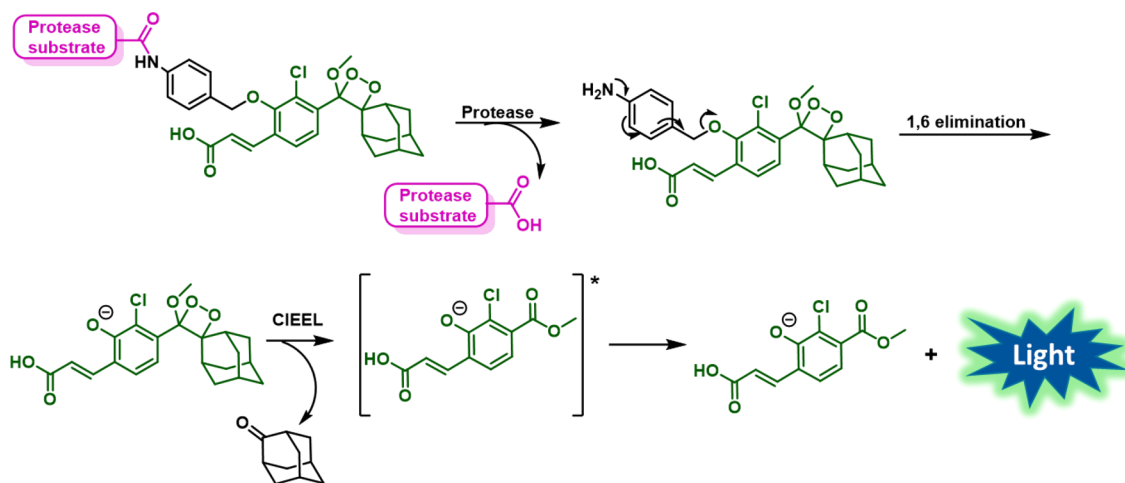


Figure 2. General activation and chemiexcitation pathway of protease chemiluminescent probes.

In 2002, the Ellman group reported the synthesis of a Rink Amide AM resin, immobilized with 7-amino-4-carbamoylmethylcoumarin fluorescent dye.¹⁵ The amino functional group of the dye was used as a handle to perform SPPS. The protease fluorescent probe was directly obtained after cleavage of the peptide–dye conjugate from the resin (Figure 1B). Such a simple and elegant synthetic strategy was found to be extremely efficient and enabled the straightforward preparation of PS-SCLs. Over the years, this methodology has proven to be particularly useful, contributing to the discovery of numerous selective substrates and inhibitors, and thereby helping to elucidate many key biological functions of proteases.^{16–19}

The most common fluorogenic reporter used in SPPS for preparation of protease probes is the 7-amino-4-methylcoumarin dye (AMC). However, while fluorescence has some important benefits, this modality also has limitations, since the need for an external light source for excitation hampers the sensitivity of the assay. In addition, the use of AMC, which emits light in the blue region, is not optimal for imaging applications due to the low tissue-permeability of short wavelengths. Recently, our group developed chemilumi-

nescent luminophores that are highly emissive under physiological conditions.^{20–22} Molecular probes composed of these luminophores were found to be suitable for monitoring enzymatic activity with great sensitivity, both *in vitro* and *in vivo*.^{23–25} The general design and activation pathway for such chemiluminescent probes is illustrated in Figure 2. Remarkably, protease probes based on our chemiluminescent dioxetane luminophore exhibited up to 16,000-fold higher sensitivity in comparison to analogous fluorescent probes based on AMC dye.^{26–31}

Although chemiluminescent probes for proteases showed unprecedented detection sensitivity, their synthesis, which is performed in solution and involves multiple purification steps, poses a major limitation for obtaining a diverse substrate scope. To overcome this limitation, we report here a general solid-phase synthesis approach to prepare chemiluminescent protease probes by peptide elongation on an immobilized chemiluminescent precursor. The establishment of such a platform could contribute immensely to obtaining positional libraries for protease chemiluminescent probes, suitable for a wide range of applications.

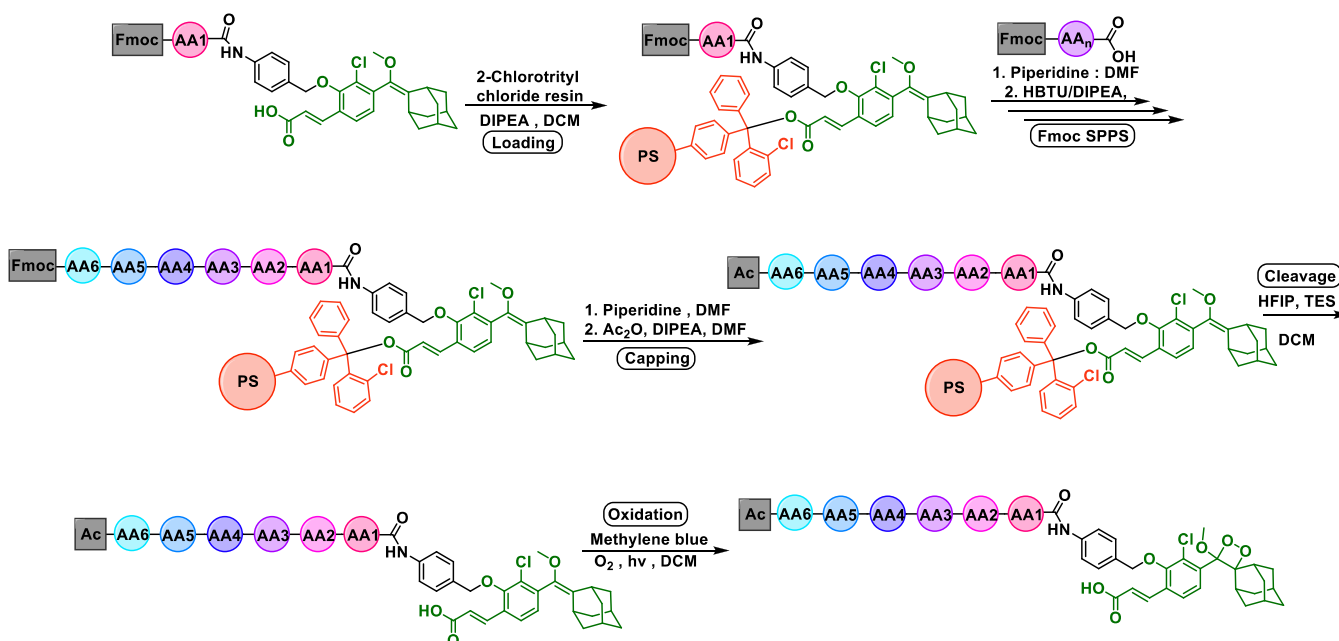


Figure 3. Schematic solid-phase synthesis of protease probes using an anchored chemiluminescent precursor (enol-ether) on 2-chlorotrityl-chloride resin.

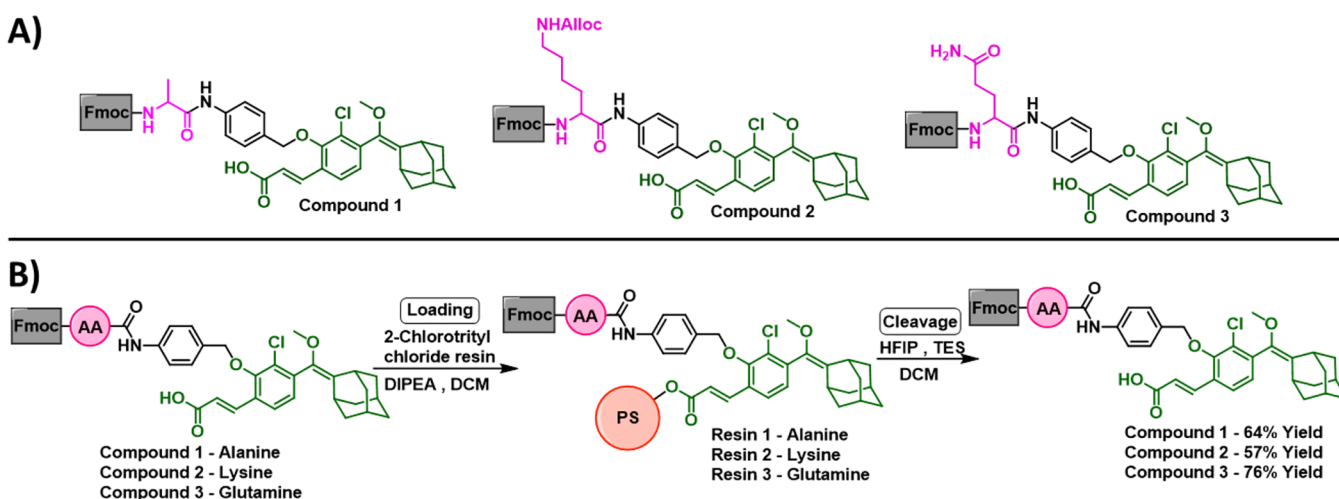


Figure 4. (A) Chemical structures of ready-to-load enol-ethers attached with the Fmoc-amino acids: alanine, lysine, and glutamine. (B) Synthesis of resins 1, 2, and 3 and their loading efficiency.

The general layout of our approach is described in Figure 3. Our methodology relies on the initial preparation of a phenol enol-ether (dioxetane precursor) building block, attached through a *p*-aminobenzyl alcohol (PABA) linker to the C-terminus of the first Fmoc-protected amino acid (Fmoc-AA) of the desired sequence. This building block is then immobilized on a 2-chlorotrityl-chloride resin through the acrylic acid substituent by an acid-labile ester linkage. Next, a stepwise elongation of the peptide is performed using standard Fmoc SPPS. After cleavage of the peptide-enol-ether from the resin, a simple oxidation step is performed to generate the final dioxetane protease probe. The immobilization of the enol-ether precursor on 2-chlorotrityl-chloride resin allowed us to perform the cleavage step by using the mild acid, hexafluoro-*iso*-propanol (HFIP). Such a mild acid can selectively cleave the trityl-ester linkage, while keeping the relative unstable enol-ether functionality intact.

The synthetic pathway for the preparation of an enol-ether building block, attached to one Fmoc-AA, is described in the Supporting Information (see Scheme S1). Following this synthetic route, we synthesized building blocks 1, 2, and 3 (Figure 4A), enol-ethers preloaded with alanine (Ala), lysine (Lys), and glutamine (Gln). When Lys was applied, an Alloc protecting group was used to protect the ϵ -amino side chain (see SI for synthetic route). We chose to focus on the amino acids Ala, Lys, and Gln, since they are present in the peptidyl substrate P1 position of several known proteases.

To test our strategy, the loading of building blocks 1, 2, and 3 on the 2-chlorotrityl-chloride resin, using *N,N*-diisopropylethylamine (DIPEA) as a base and dichloromethane (DCM) as a solvent, was followed by a capping step using a solution of MeOH, DIPEA, and DCM. After several rounds of washing, the resin was treated with the cleavage cocktail. This step was followed by recovery and purification of the starting materials

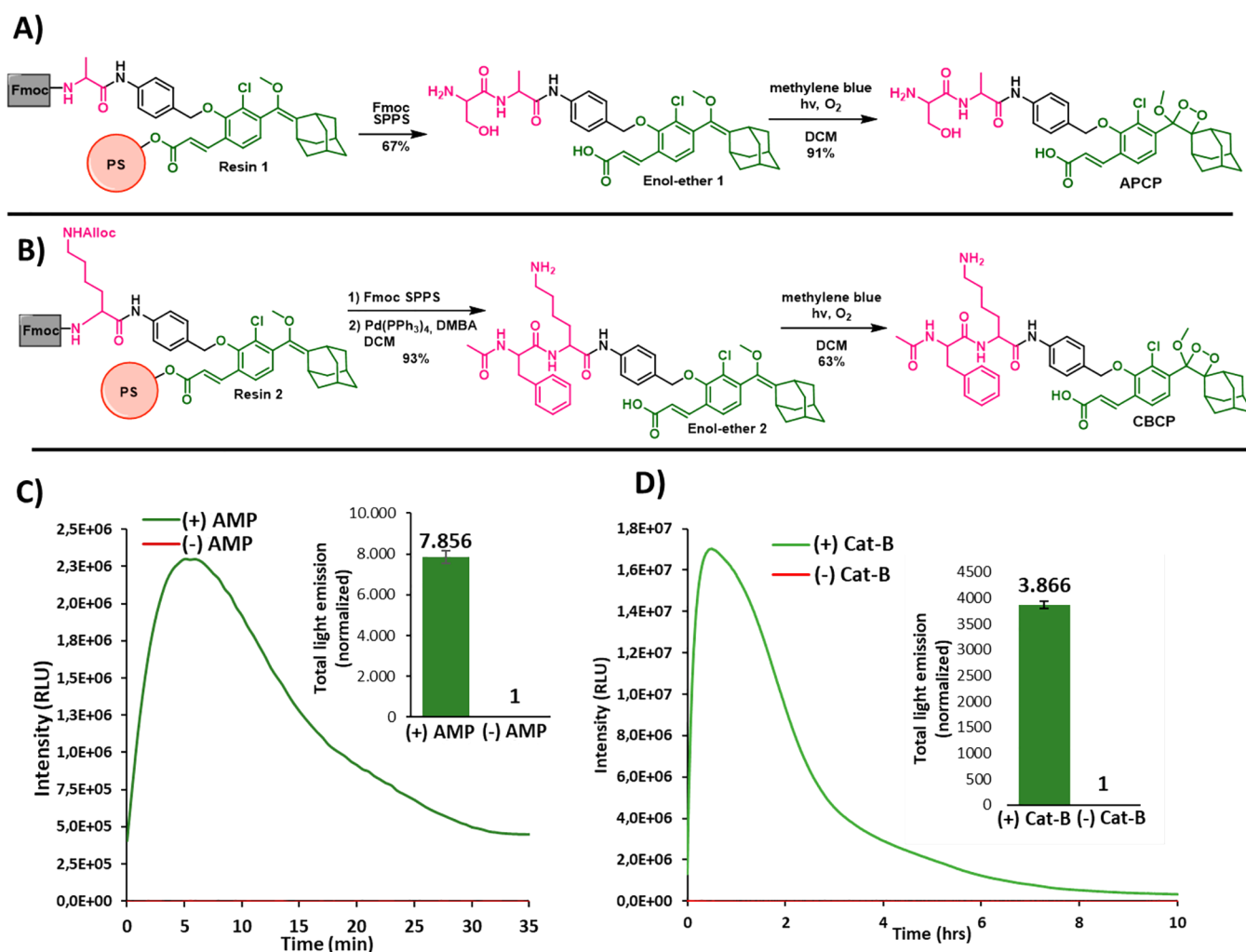


Figure 5. (A) Synthetic route to prepare probe APCP. (B) Synthetic route to prepare probe CBCP. (C) Light-emission profile and total light emission of probe APCP [10 μM] in the presence and absence of AMP (aminopeptidase M, porcine kidney, [20 nM]), 1% DMSO, in PBS, pH = 7.4. (D) Light-emission profile and total light emission of probe CBCP [50 μM] in the presence and absence of Cat-B (cathepsin B, human liver, [1.4 U/mL]), 1% DMSO, in activity buffer (0.1 M PBS [1.37 M], KCl [27 mM], EDTA [1 mM], glutathione [5 mM]).

(building blocks 1, 2, and 3). The final yields of this process are depicted in Figure 4B. All three building blocks were loaded and cleaved with decent yields. This initial evaluation provided a clear indication that the enol-ether functionality is compatible with the loading and cleavage conditions.

With the resin-immobilized enol-ethers in hand (Resins 1, 2, and 3), we sought to evaluate our approach, to prepare chemiluminescent protease probes through solid-phase synthesis. For simplicity, we initially chose to prepare protease chemiluminescent probes equipped with simple dipeptidyl triggering substrates. Two such probes were prepared. The first one, with the dipeptidyl substrate Ser-Ala, is a probe for detection of aminopeptidase (AMP) proteolytic activity.³² SPPS using Resin 1 afforded Enol-ether 1 in high purity with a yield of 67% after cleavage. Oxidation of this enol-ether by singlet oxygen afforded the aminopeptidase chemiluminescent probe (APCP) in 91% yield (Figure 5A). Overall, APCP was synthesized using SPPS with a total yield of 61% and with high purity (SI Figure S10). The second probe that was prepared by this method, with a dipeptidyl substrate, is a probe aimed for activation by the protease cathepsin B (Cat-B). Out of several known peptidyl substrates of Cat-B, we chose to focus on the dipeptidyl substrate Ac-Phe-Lys, which was demonstrated in

numerous examples of imaging applications.^{8,33–35} SPPS using Resin 2, followed by cleavage and removal of the Alloc protecting group using Pd⁰, afforded Enol-ether 2 in 93% yield. Oxidation of this enol-ether by singlet oxygen gave the Cat-B chemiluminescent probe (CBCP) in 59% yield after purification (Figure 5B). Overall, probe CBCP was synthesized using SPPS with a total yield of 63% and with high purity (SI Figure S12).

The light emission profiles of chemiluminescent protease probes APCP and CBCP were measured in the presence and in the absence of the proteases AMP and Cat-B, respectively. Probe APCP in PBS 7.4 showed almost no light emission (Figure 5C). However, in the presence of AMP, a clear increase of signal was detected, displaying a typical chemiluminescent kinetic profile of rise and decay. Remarkably, the total light emitted signal by the probe with AMP was about 7800 times stronger than the signal produced in the absence of the protease.

The light emission profiles of probe CBCP obtained in the presence and in the absence of Cat-B are shown in Figure 5D. Likewise, Cat-B was able to activate the probe with an initial sharp increase of the light emission signal, followed by gradual decay. As expected, almost no light emission was observed in

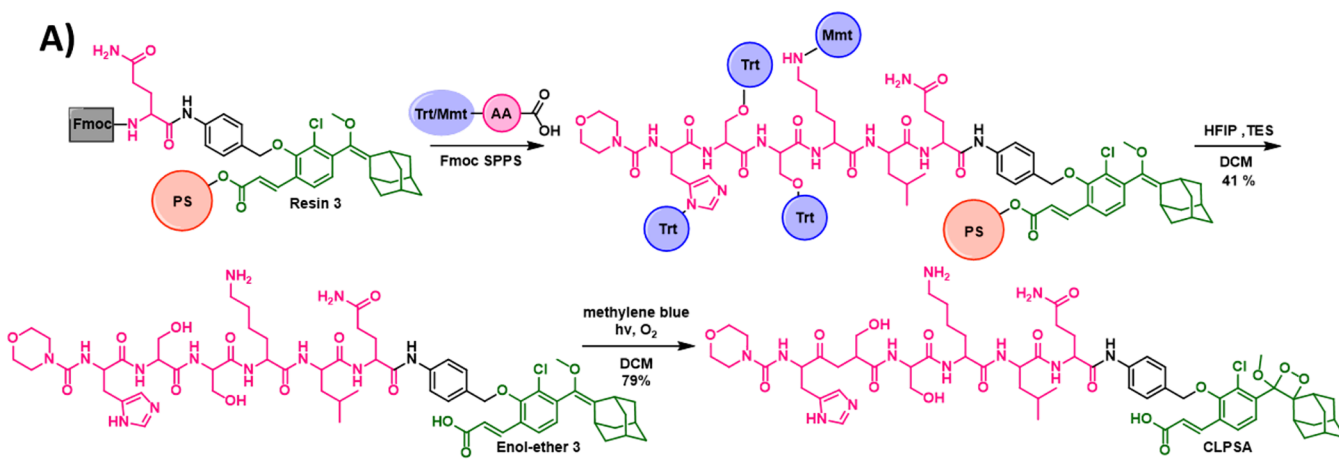


Figure 6. (A) Synthetic route to synthesize **CLPSA** probe using SPPS with trityl protected amino acids. (B) Kinetic profile and total light emission of **CLPSA** [$10 \mu\text{M}$] in the presence and absence of PSA (prostate specific antigen from human seminal fluid, [$10 \mu\text{g}/\text{mL}$]), 2% DMSO, in PBS, pH = 7.4.

the absence of Cat-B, and the signal-to-noise ratio value produced by probe **CBCP** was 3800-fold.

To apply our solid-phase synthetic methodology to a broader scope of protease substrates, we sought to demonstrate a more complex example that incorporates multiple amino acids, including ones that require protecting groups on their side chains. Since the final peptide-enol-ether is cleaved from the 2-chlorotrityl resin by HFIP, we assumed that commercially available trityl-protected amino acids will be suitable for use in our solid-phase synthesis. To validate this hypothesis, we designed a synthetic route to prepare a chemiluminescent probe for the detection of Prostate-Specific Antigen (PSA) (Figure 6A), which was previously prepared by our group via standard synthesis in solution.²⁷

The peptidyl substrate of probe **CLPSA** is composed of the sequence Mu-HSSKLQ. This peptide has four amino acids with side residues that require protecting groups during the solid-phase-synthesis. Ideally, those protecting groups should be removed under the same conditions required for the final cleavage. To test the feasibility of our methodology for preparing probe **CLPSA** by solid-phase-synthesis, we used resin 3 as a starting material and commercially available trityl (Trt) protected His and Ser amino acids. In the case of Lys, we chose the monomethoxytrityl (Mmt) as a protecting group for the ϵ -amino side chain. The solid-phase-synthesis of probe **CLPSA** enol-ether precursor was achieved through six sequential coupling steps starting from resin 3. Cleavage by HFIP and subsequent oxidation with singlet oxygen afforded

probe **CLPSA** in 41% yield after RP-HPLC purification. Importantly, the four trityl-based protecting groups were efficiently removed from the peptide substrate during the cleavage of the enol-ether precursor from the resin by HFIP.

This example effectively demonstrates that chemiluminescent protease probes can be conveniently synthesized using our solid-phase-synthetic methodology, even when complex peptidyl substrates are required. Although **CLPSA** can be prepared by a solution-phase approach, the solid-phase-synthetic approach is considerably simpler and consumes less time and resources. The light-emission profile of **CLPSA** was then evaluated in the presence and in the absence of PSA. Upon addition of PSA, probe **CLPSA** was able to produce light-emission signal 40 times greater than that observed in PBS 7.4 alone (Figure 6B). The observed steady-state signal is attributed to the relatively low enzymatic activity of PSA.²⁷

The method developed in this work, includes an oxidation step, by singlet oxygen, of the peptide-enol-ether intermediate to its corresponding dioxetane. Therefore, an amino acid like methionine (Met) can also be oxidized to its sulfoxide derivative. To clarify the limitations of the synthetic method, we subjected peptides, that include the amino acids Met or Trp, to singlet oxygen conditions. Indeed, oxidation with singlet oxygen of a Met-based conjugate, composed of the sequence, Fmoc-Met-Ala-PABA-Enol-ether, has resulted in the corresponding dioxetane and the sulfoxide derivative for the Met. Oxidation with singlet oxygen of a Trp-based conjugate, composed of the sequence Fmoc-Trp(Boc)-Ala-PABA-Enol-

ether, has resulted in the corresponding dioxetane with no side oxidation of the Trp. The Boc protecting group can be removed from the Trp, under mild acidic conditions, after the oxidation of enol-ether to the dioxetane. In addition, subjection of Fmoc-Trp to singlet oxygen has confirmed the stability of this amino acid to the oxidation conditions (see Supporting Information Figures S3–S8).

In summary, we developed a practical and efficient solid-phase synthetic methodology for the general preparation of protease chemiluminescent probes. To validate the applicability of the methodology, we have preloaded 2-chlorotriyl-chloride resin with three chemiluminescent enol-ether precursors, each attached to a different Fmoc-AA. Two of these preloaded resins were used in the preparation of two chemiluminescent probes, by the solid-phase synthesis, with dipeptidyl substrates designed for activation by the AMP and Cat-B proteases. The third preloaded resin, was applied for the synthesis of a chemiluminescent probe aimed for detection of PSA, which is composed of a relatively complex peptidyl substrate. This peptidyl substrate, is comprised of six amino acids, four of which require the use of protecting groups on their side chains. The cleavage conditions of the peptide-enol-ether precursor from the trityl resin by HFIP, were compatible with the removal of the protecting groups and the stability of the enol-ether functionality. We anticipate that the described methodology would be useful for the preparation of vast and diverse peptidyl substrates for chemiluminescent protease probes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.bioconjchem.1c00384>.

(Synthetic procedures, characterization data (NMR and MS) for all new compounds and chemiluminescence control experiments PDF)

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Notes

The authors declare no competing financial interest.

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