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Chemiluminescence Molecular Probe with a Linear Chain Reaction Amplification Mechanism

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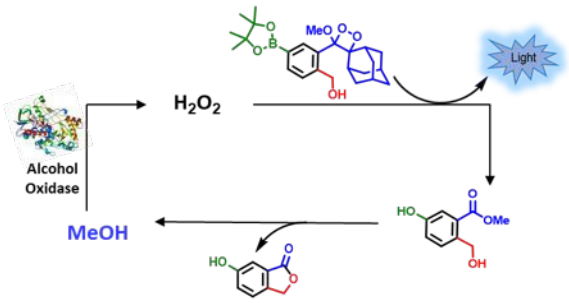
Abstract: A new signal amplification probe with a linear chain reaction amplification mechanism and distinct chemiluminescence output was developed. The probe is composed of a unique structural motif that combines a chemiexcitation mechanism with an intramolecular transesterification into a single molecular structure. As demonstrated with a probe designed to detect hydrogen peroxide, an auto-inductive chemiluminescence signal amplification was obtained through methanol release by an intramolecular transesterification of the generated 2-hydroxymethylbenzoate derivative. The methanol was then oxidized by alcohol oxidase to regenerate the analyte of interest, hydrogen peroxide. Our probe enabled direct measurement of light emission with a limit of detection of 2.5 µM, whilst the assay was rapidly completed within 14 to 150 minutes. Such molecular probes with chemiluminescence signal enhancement through analyte amplification could be used for the detection of other chemical and biological analytes.

Graphical Abstract



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Signal amplification techniques are widely used to enhance the detection sensitivity of analytes and enzymes for diagnostic purposes.[1, 2] The ability to detect certain analytes with high sensitivity is of great importance in various scientific fields where rapid analyte detection is required.[3, 4] Thus, there is an unmet need for detection techniques with improved sensitivity and selectivity via signal amplification modes.

Over the last 10 years, several new signal amplification approaches have been developed.[5-8] One approach to obtain signal amplification relies on an auto-inductive mechanism that produces a detectable optical output. Several groups, including ours, have demonstrated new concepts to achieve auto-inductive signal amplification based on chemical reactions with small molecules.[9-13] We have developed a unique modular technique for exponential amplification of a diagnostic signal; amplification is achieved through a dendritic chain reaction, generated by the disassembly of self-immolative dendrimers.[10, 14-16] A single activation event initiated by the analyte of interest leads to a chain reaction that ultimately results in complete disassembly of all probe molecules and release of many copies of a reporter molecule. Taking advantage of the modular design of the probe, our group and others have applied the dendritic chain reaction technique for the detection of various analytes.[11, 14, 17-20] In order to follow the progress of the signal amplification, an optically detectable dye is commonly used as a chromogenic reporter.

Fluorescence and visible-absorbance chromogens are among the most useful reporters for optical sensing.[8, 21-24] However, chemiluminescence is considered as the most sensitive modality for detection due to the high signal-to-noise ratio obtained in such assays.[25-35] Chemiluminescence is the conversion of chemical energy into the emission of visible light as a result of a chemical reaction that leads to chemiexcitation. Very recently, we have developed

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an effective molecular building block that intrinsically integrates a chemiluminescence turn-ON phenomenon into a self-immolative domino-like fragmentation mechanism.[36, 37] This type of self-immolative chemiluminescence building block can execute the dual functions of quinone-methide elimination and chemically initiated electron exchange luminescence. Using this building block, a new signal amplification system with an auto-inductive mode of action and distinct chemiluminescence output was also demonstrated.[38] Understanding the relationship between structure and function can assist in design of new amplification systems with improved sensitivity and selectivity. Here we describe the use of a new type of molecular building block for design and synthesis of a chemiluminescence probe with a linear chain reaction (LCR) amplification mechanism.

It is well known that 2-hydroxymethylbenzoate esters are unstable under neutral and basic aqueous conditions due to an intramolecular trans-esterification reaction between the benzylic alcohol and the alkyl benzoate ester.[39, 40] This reaction occurs through intramolecular nucleophilic attack of the benzylic alcohol on the ortho-neighboring alkyl benzoate group and affords the corresponding phthalide while releasing an alkoxy moiety. As presented in Figure 1A, the byproduct of the chemiexcitation process of Schaap's 1,2-dioxetane **I**, is the alkyl benzoate ester **III**. We therefore sought a molecular design that would generate a 2-hydroxymethylbenzoate derivative from Schaap's 1,2-dioxetane. We reasoned that this could be implemented through a benzylic alcohol substituent on the original Schaap's 1,2-dioxetane (Figure 1B). The triggering of dioxetane **V** leads to the generation of 2-hydroxymethylbenzoate **VIII**, which can then undergo an intramolecular cyclization reaction to release an alkoxy group and simultaneously generate phthalide **IX**. The release of the alcohol molecule occurs by intramolecular cyclization, and this reaction can be harnessed to generate a reagent with

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identical reactivity to that of the analyte of interest. Such a pathway will produce a sensor with an auto-inductive amplification mechanism.

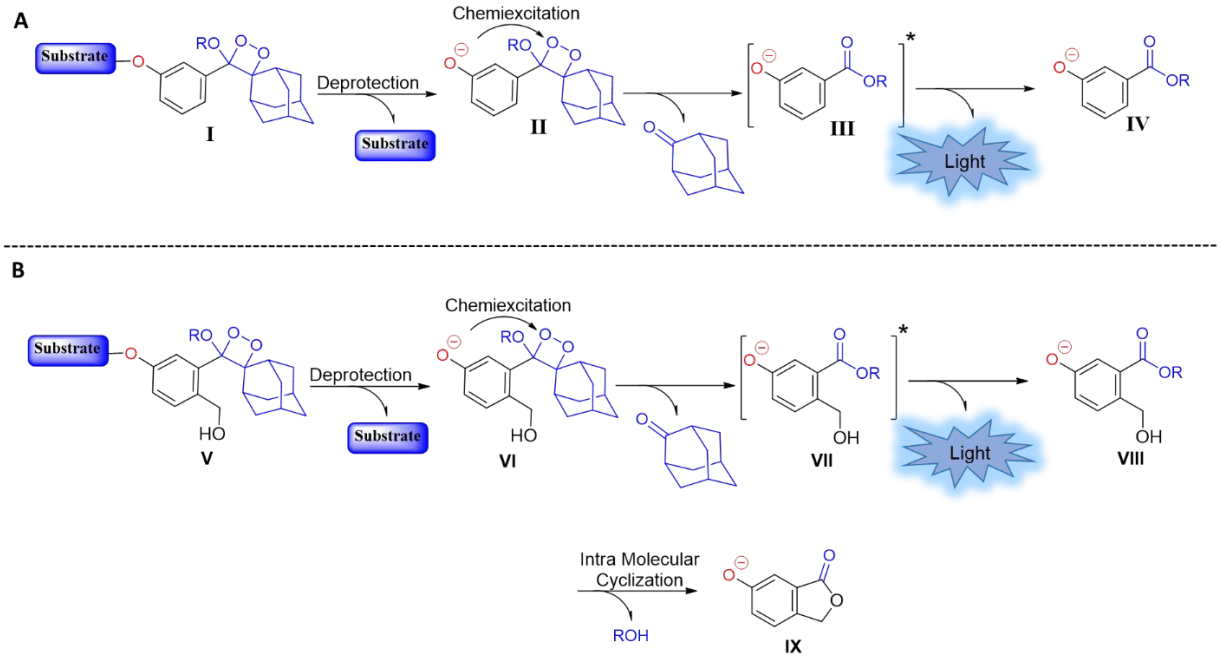

Figure 1: Molecular structure and activation pathway of (A) triggerable 1,2-dioxetane **I** and (B) triggerable 1,2-dioxetane **V**; the latter reacts through an intramolecular trans-esterification reaction.

Probe **1,** designed to detect the analyte hydrogen peroxide, consists of phenoxy 1,2-dioxetane as a chemiluminescent reporter and phenylboronic ester as a triggering substrate for hydrogen peroxide (Figure 2). The LCR amplification cycle of the probe is illustrated in Figure 2B. Cleavage of the trigger of probe **1** by hydrogen peroxide under alkaline conditions leads to the emission of chemiluminescent blue light and the generation of phenolate intermediate **1c**. The latter undergoes intramolecular trans-esterification to release a methanol molecule. The released methanol is then oxidized by alcohol oxidase (AOX) and molecular oxygen to generate formaldehyde and hydrogen peroxide, which can then activate an additional probe

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molecule. The assimilation of both chemiexcitation and analyte regeneration processes in one single molecular moiety is expected to provide an effective probe for a chemiluminescence amplification cycle.

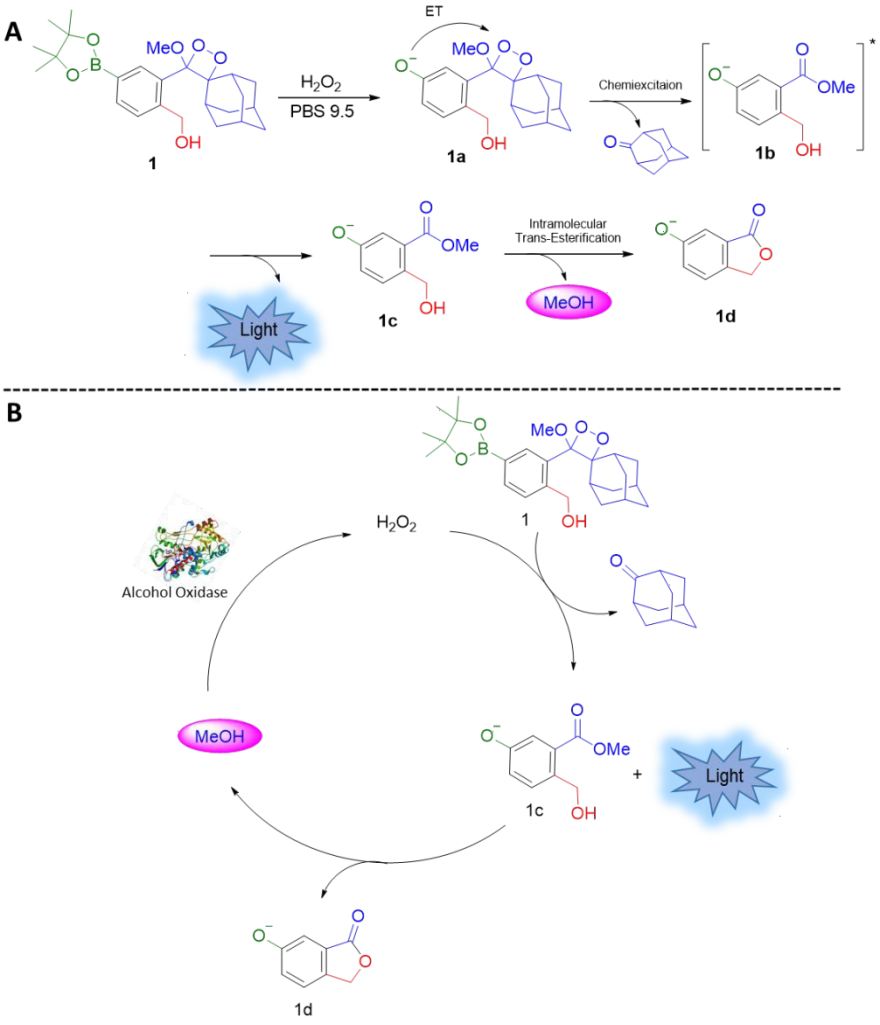

Figure 2: (A) Chemical structure and disassembly mechanism of probe **1**. (B) Auto-inductive disassembly and chemiluminescence emission of probe **1** triggered by hydrogen peroxide.

The synthesis of probe **1** was achieved as described in the Supporting Information section. The amplification kinetic profiles of probe **1** (250 μM) incubated with AOX (1 EU) and different concentrations of hydrogen peroxide (0-250 μM) in carbonate buffer (pH 9.5) was monitored

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by chemiluminescence emission at 499 nm (Figure 3A). Typical light emission profiles were obtained for all solutions with signal increase to a maximum followed by decay to zero. In a low concentration of hydrogen peroxide, a longer time period was required to achieve maximum chemiluminescence intensity. When 1.0 equivalent (250 μM) of hydrogen peroxide was applied, the chemiluminescence system reached a maximum intensity within 14 minutes compared to 23 minutes for 0.3 equiv., 36 minutes for 0.1 equiv., 50 minutes for 0.01 equiv., and 64 minutes in the absence of hydrogen peroxide. The total light emission values were identical for all hydrogen peroxide concentrations tested (Figure 3B).

The light emission signal generated in the absence of hydrogen peroxide was also amplified as a result of spontaneous side reactions. However, the observed background signal was substantially lower than the signal obtained for a sample treated with 0.01 equiv. of hydrogen peroxide. Within the time frame of the measurements (200 min), the background signal reached a maximum of about half that of the signal generated in the presence of hydrogen peroxide. After approximately 12 hours, the background signal reached maximum intensity (data not shown). Given the noise generated by this background signal, the lowest detectable hydrogen peroxide concentration was found to be 2.5 μM.

For a control experiment, we synthesized probe **2**; an analogues probe that cannot produce an amplified signal. This probe has a pinacol-boron group that serves as a triggering substrate for hydrogen peroxide. The benzylic alcohol is protected with a methyl group to prevent an intramolecular trans-esterification reaction. Thus, probe **2** emits the chemiluminescent reporter in an equal molar ratio to the applied concentration of the hydrogen peroxide analyte. The increased sensitivity of the LCR technique with probe **1**, in comparison to this classic probe **2**, was demonstrated when the background noise was subtracted from the

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measured signals and signal obtained were compared (Figure 3D). In the presence of 1.0 equivalent of hydrogen peroxide, probe **2** generated signal intensity identical to that of probe **1**. However, in the presence of less hydrogen peroxide, the signals measured by probe **1** were significantly larger than those obtained by probe **2**. The ratio of the auto-inductive signal, obtained by dividing the signal from probe **1** to that obtained from probe **2**, increased with decreasing analyte concentration. In the detection limit of our assay sensitivity, using 0.01 equivalents of hydrogen peroxide, the signal obtained by probe **1** was about 19-fold brighter than that obtained using probe **2**.

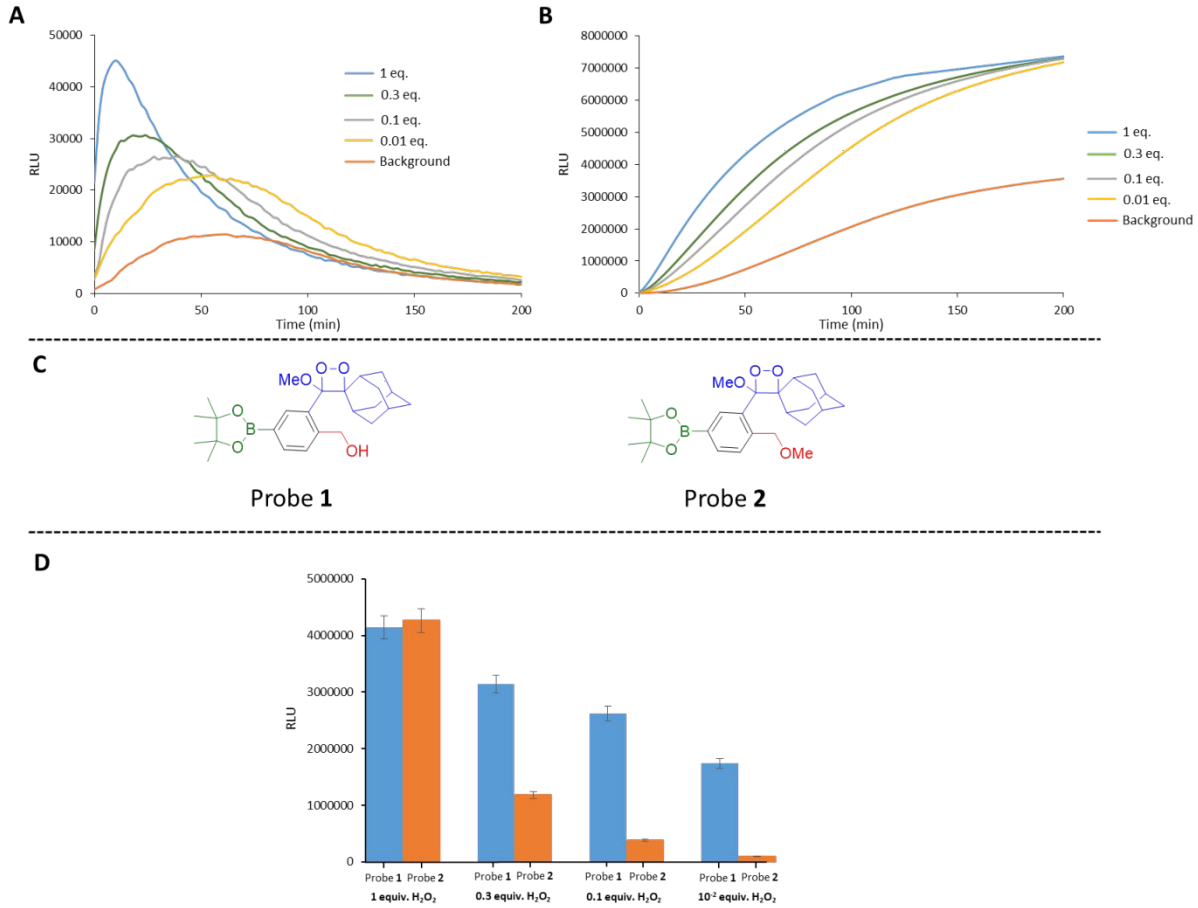

Figure 3: (A) Kinetics of chemiluminescent signal from probe **1** (250 μM) with various concentrations of H_2O_2 (0, 2.5, 25, 83, and 250 μM) in the presence of 1 EU AOX in buffer of

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pH 9.5. B) Total signal as a function of time from probe **1** with various concentrations of H_2O_2 (0, 2.5, 25, 83, and 250 μM) in the presence of 1 EU AOX in buffer of pH 9.5. (C) Chemical structures of probes **1** and **2**. (D) Comparison of signals measured from probe **1** (LCR amplification technique, blue) vs. signals measured using control probe **2** (green). The background signal present at 72 min was subtracted from the values shown.

To test whether the amplification cycle indeed occurs as proposed in Figure **2**, we monitored the disassembly profile of probe **1** over time by a comparative reversed-phase HPLC technique. Figure 4 shows three HPLC chromatograms recorded at different times after the addition of probe **1** to hydrogen peroxide (0.01 equiv.) and AOX (1 EU) in buffer of pH 9.5. Before pinacol-borane oxidation, the HPLC spectrum of probe **1** shows one characteristic peak with a retention time of 20 minutes (Fig. 4A). At 130 minutes after addition of hydrogen peroxide, the chromatogram shows three peaks (Figure 4B): probe **1**, benzoate intermediate **1c** (retention time; 6.5 minutes) and phthalide **1d** (retention time; 7.5 minutes). The complete disassembly of probe **1** was achieved within 210 minutes (Fig. 4C). This time course of disassembly is in good agreement with the kinetic profile obtained by monitoring of the chemiluminescence (Fig. 3B). Furthermore, these results demonstrate that complete disassembly of probe **1** is achieved in the presence of 0.01 equiv. of the hydrogen peroxide analyte.

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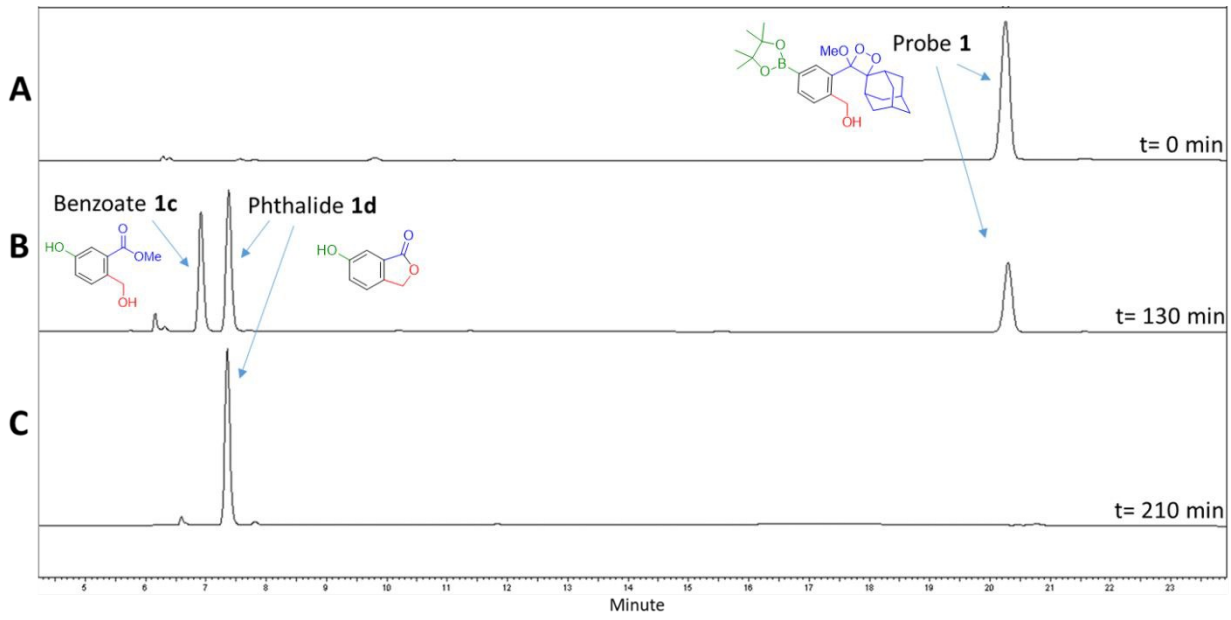

Figure 4: The progression of the autocatalytic disassembly of probe **1** (250 µM) upon addition of hydrogen peroxide (2.5 µM) in buffer (pH 9.5) monitored using RP-HPLC (λ = 270 nm).

Mechanistically, most of the previously reported chemiluminogenic probes of hydrogen peroxide react with a single hydrogen peroxide molecule and release a single chemiluminogenic probe molecule.[41-43] Recently, auto-inductive signal amplification techniques involving chemiluminescence have been reported. Turan's group described the attachment of Schaap's dioxetane to an AB_2 self-immolative dendron. Such a probe shows a moderate chemiluminescence signal amplification (only 2-fold) with a detection-limit of the analyte of around 240 µM.[44] Akkaya and co-workers reported a self-immolative dendritic probe for amplification of chemiluminescent signal based on dendritic chain reaction.[35] This self-immolative dendron was equipped with two dioxetane moieties as reporters, two fluoride units attached through benzylic group, and a dimethyl-tertbutylsilyl group as a triggering substrate for fluoride. Similarly, Hisamatsu *et al*. described a chemiluminescent amplification cycle based on a two-component dendritic chain reaction system.[28] The chemiluminescence

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molecular probe **1** described in this work enabled direct measurement of light emission with a limit of detection of 2.5 μM. Furthermore, the assay was completed within 14 to 150 minutes.

The reported molecular design for a probe with chemiluminescence signal enhancement through analyte amplification could be applicable for the detection of other chemical and biological analytes. The latter could be achieved by introduction of various analyte-responsive groups as triggering substrates and conversion the methoxy leaving group (here a methyl ester) to the appropriate triggering analyte such as methyl thiol or fluoride.

In summary, we have developed a new signal amplification system with an LCR mode of action and distinct chemiluminescence output. The molecular system combines a chemiexcitation mechanism with an intramolecular transesterification into a single building block. As demonstrated with the probe designed to detect hydrogen peroxide, LCR chemiluminescence signal amplification was obtained through methanol release and *in situ* oxidation by an intramolecular transesterification of the generated 2-hydroxymethylbenzoate derivative. A kinetic experiment analyzed by RP-HPLC provided support for the proposed amplification mechanism. This type of molecular probe with chemiluminescence signal enhancement through analyte amplification could also be used for the detection of other analytes.

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