Biocompatible Flash Chemiluminescent Assay Enabled by Sterically Hindered Spiro-Strained-Oxetanyl-1,2-Dioxetane

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Abstract

Chemiluminescence is the emission of light that occurs as a result of a chemical reaction. Depending on the rate of chemiexcitation, light emission can occur as a long-lasting, low-intensity, glow-type reaction or a rapid, highly intense flash-type reaction. Assays using a flash-type mode of action provide enhanced detection sensitivity compared to those using a glow-type mode. Recently, our group discovered that applying spiro-strain to 1,2-dioxetanes significantly increases their chemiexcitation rate, thereby transforming glow-type chemiluminescence into flash-type chemiluminescence. However, further examination of the structure-activity relationships revealed that the spiro-strain severely compromises the chemical stability of the 1,2-dioxetanes. We hypothesized that a combination of spiro-strain, steric hindrance, and an electron-withdrawing effect, will result in a chemically stable spiro-strained dioxetane with an accelerated chemiexcitation rate. Indeed, spiro-fused tetramethyl-oxetanyl exhibited a 128-fold faster chemiexcitation rate compared to adamantyl while maintaining similar chemical stability, with a half-life of over 400 hours in PBS 7.4 buffer at room temperature. Turn-on probes composed of tetramethyl-oxetanyl spiro-dioxetane exhibited significantly improved chemical stability in bacterial and mammalian cell media compared to previously developed dioxetane probes fused to a cyclobutyl unit. The superior chemical stability enables a tetramethyl-oxetanyl dioxetane probe to detect β-gal activity with enhanced sensitivity in *E. coli* bacterial assays and leucine aminopeptidase activity in tumoral cell lines. Overall, the development of the tetramethyl-oxetanyl dioxetane luminophore enables us to enhance the detection sensitivity of chemiluminescent probes while maintaining high chemical stability. The results obtained in this study should assist in designing of improved chemiluminescent probes and underscore the significance of strain-release techniques in enhancing the detection sensitivity of chemiluminescence assays.

TOC Graphics
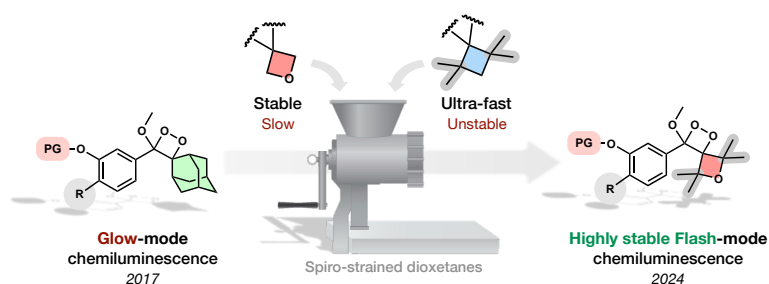
Introduction

In the past three decades, chemiluminescent probes have emerged as versatile and powerful molecular tools for various applications.[1-3] Unlike fluorescence, in chemiluminescence, light emission occurs as a result of a chemical reaction. This distinct advantage results in exceptional sensitivity since the need for external light irradiation, which induces autofluorescence and light scattering, is eliminated.[4] The development of modular turn-on chemiluminescent probes began in 1987 with the discovery of triggerable phenoxy-1,2-dioxetanes (Figure 1A).[5] By masking the phenol with a substrate for a specific enzymatic activity, chemiluminescent probes can be tailored to detect a variety of enzymatic activities. Upon removal of the substrate group, a chemiexcitation process is initiated, leading to the formation of an excited benzoate. This excited benzoate subsequently decays to its ground state, resulting in light emission. Depending on the rate of chemiexcitation, light emission can occur by either a slow glow-type reaction or a fast flash-type reaction. Glow-type chemiluminescence is characterized by a long-lasting light emission profile with relatively low intensity since only a small number of molecules undergo chemiexcitation at any given time. In contrast, flash-type chemiluminescence involves rapid chemiexcitation, producing a bright burst of light that is completed in a matter of seconds or minutes. Accordingly, assays employing a flash-type mode of action provide enhanced detection sensitivity compared to those employing a glow-type mode.[6]

Recently, our group discovered a unique strategy to enhance the chemiexcitation rate of dioxetane luminophores, thereby converting glow-type chemiluminescence into flash-type chemiluminescence.[7] By replacing the conventional spiro-adamantyl with spiro-cyclobutyl (Figure 1B), angular spiro-strain is generated, inducing a spring-loading effect on the peroxide O-O bond. This strain effect accelerates the dioxetane decomposition into an excited state, thereby significantly increasing the chemiexcitation rate. This discovery enabled rate acceleration without a decrease in the quantum yield, generating extremely bright dioxetanes with unprecedented detection sensitivity. Historically, chemists have been cautious about replacing the conventional spiro-adamantane unit with alternative substituents, due to concerns associated with the chemical stability of the dioxetane and the formation of ene-products during oxidation.[8] The ene side product is obtained through the elimination of a proton located at the allylic position of the enolether.[9] In both cyclobutyl and adamantyl enolethers, the formation of this undesired ene-product is entirely prevented because the elimination reaction results in the formation of a highly constrained cyclic alkene. Although the formation of the ene-product is prevented in the spiro-cyclobutyl, an examination of structure-activity relationships revealed that applying spiro-strain on 1,2-dioxetanes severely compromises their chemical stability. While the stability of spiro-strained cyclobutyl-dioxetanes

proved sufficient for prolonged storage at low temperatures, in a cell-growth media environment, they readily decomposed. In quest of developing a stable, biocompatible spiro-strained dioxetane with flash-type chemiluminescence, we hypothesized that combining spiro-strain, steric hindrance, and an electron-withdrawing effect could yield a highly stable spiro-strained dioxetane with accelerated chemiexcitation mode. (Figure 1C). Here we report the development of the first biocompatible spiro-strained dioxetane which paves the way for employing flash-type chemiluminescence across a range of biological applications.
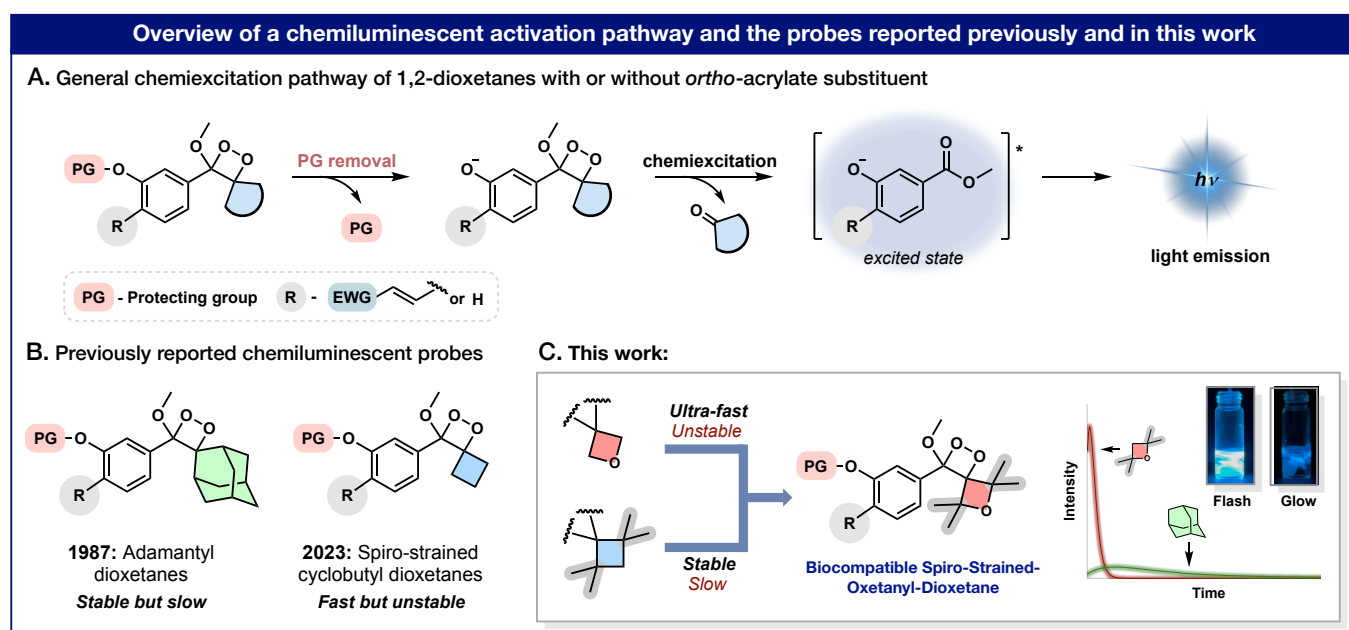

Figure 1. (A) Activation and chemiexcitation pathway of 1,2-dioxetanes. (B) General structure and characteristics of adamantyl and cyclobutyl substituted dioxetanes. (C) This work: Sterically hindered spiro-strained oxetanyl 1,2-dioxetane.

Results and Discussion

To properly examine the effects of spiro-strain, steric hindrance, and electron withdrawal by an electronegative heteroatom on the chemiexcitation rate and the chemical stability, the 1,2-dioxetane probes, prepared for this study, were masked with a *tert*-butyl-dimethylsilyl (TBS) group as a triggering substrate (Figure 2A). Consequently, their chemiexcitation could be simply initiated by the addition of fluoride (TBAF). The relative chemiexcitation rates of the studied dioxetanes were calculated by measuring their total light emission $t_{1/2}$ values according to the plots presented in Figure 2B and Figure S1-S2 in the Supporting Information.

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As previously reported[7], due to the spiro-strain effect, the chemiexcitation of cyclobutyl-1,2-dioxetane, TBS-**CB**, was significantly accelerated (106-fold) compared to its adamantyl counterpart, TBS-**AD**. Interestingly, the addition of an electronegative heteroatom, oxygen, into the four-member ring crafted a dioxetane probe, TBS-**OX**, with a chemiexcitation rate that was 2662-fold faster than that of TBS-**AD**. However, the chemical stability of the spiro-strained dioxetane probes, TBS-**CB** and TBS-**OX**, was sustainably compromised compared to the adamantyl, TBS-**AD**. On the other hand, the substitution of the spiro-cycloalkyl with four methyl groups, produced a dioxetane probe, TBS-**Tet**, with a chemiexcitation rate that is only 11-fold faster than that of TBS-**AD** but with much better chemical stability. The $t_{1/2}$ value measured for TBS-**Tet** in PBS 7.4 buffer at room temperature was larger than 400 hours, compared to only 4 hours for TBS-**OX**.[7]

The rate enhancement of an electron-withdrawing spiro-heterocycle and the improved stability of the sterically hindered tetramethyl-cyclobutyl motivated us to synthesize a dioxetane that combines these structural motifs, in order to gain an optimized dioxetane probe with fast chemiexcitation rate and high chemical stability. Therefore, we synthesized a new spiro-dioxetane probe, which possesses an oxetane ring with four methyl substituents. The new spiro-fused tetra-methyl-oxetanyl (TBS-**Tetrox**) exhibited a significantly faster chemiexcitation rate (128-fold) compared to that of the adamantyl, TBS-**AD**, while maintaining similar chemical stability to probe TBS-**AD**, with a half-life of more than 400 hours in PBS 7.4 buffer at room temperature (See Figure S3 in the Supporting Information). Figure 2C shows a visual demonstration of the chemiexcitation acceleration effect obtained by the TBS-**Tetrox** compared to the known spiro-adamantyl-dioxetane. These results prompted us to further evaluate the detection sensitivity and chemical stability of a dioxetane probe, equipped with spiro-fused tetramethyl-oxetanyl unit, under physiological conditions.
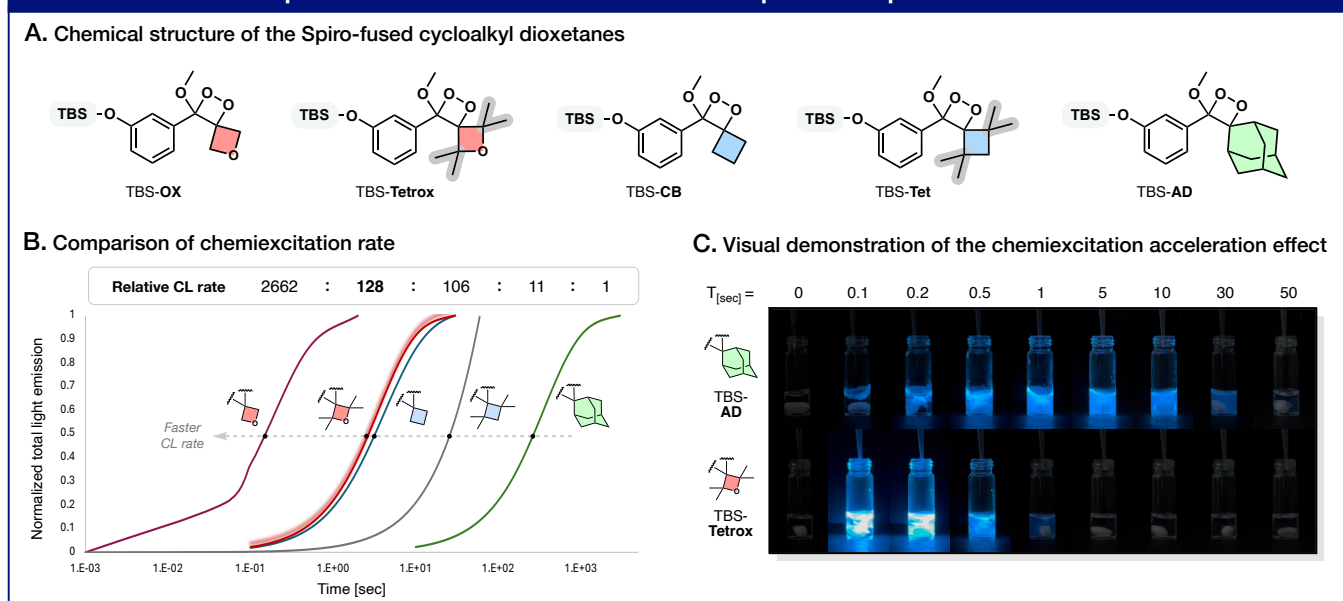
Figure 2. (A) Molecular structure of the TBS-protected cycloalkyl dioxetanes. (B) Normalized total light emission kinetic profile (time is presented in logarithmic scale). The kinetic profiles and the rate constants are presented in Figures S1-S2. (C) Visual demonstration of the light emitted by TBS-**AD** and TBS-**Tetrox** during 50 sec in the presence of TBAF in DMSO. For detailed procedures see the experimental protocols section of the supporting information.

The chemiexcitation rate acceleration observed in the phenoxy-1,2-dioxetane with spiro-fused Tetrox unit suggests that a turn-ON probe utilizing this luminophore is expected to demonstrate improved detection sensitivity compared to its adamantyl counterpart. However, to further assess the sensitivity of chemiluminescent probes with a tetramethyl-oxetanyl unit in biological assays, a modification to Schaap's dioxetane is required since their light emission in the presence of water is almost completely quenched. Several years ago, our group discovered that the incorporation of an acrylate electron-withdrawing substituent at the *ortho* position of a phenoxy-adamantyl-1,2-dioxetane chemiluminescent luminophore prevents water-mediated quenching and amplifies the light-emission intensity of the luminophore by a factor of 3000-fold.[10] Notably, this development inspired numerous research groups worldwide, including ours, to take advantage of the *ortho*-substituted phenoxy-adamantyl-1,2-dioxetane luminophore to develop useful chemiluminescent probes for various biological applications.[11-24]

Therefore, we next synthesized an *ortho*-acrylate dioxetane probe fused with a tetramethyl-oxetanyl unit, aimed for the detection of β-galactosidase activity (**β-gal-MA-Tetrox**). The ability of this probe to detect β-gal was compared against a previously reported *ortho*-acrylate dioxetane probe, fused with adamantyl unit (probe **β-gal-MA-AD**). The general molecular structure of the probes and their chemiluminescent activation pathway are presented in Figure 3A and Figure S4 in the Supporting Information. Enzymatic

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cleavage of the β-gal substrate followed by 1,6- quinone-methide elimination releases the phenoxy-1,2-dioxetane, which then undergoes chemiexcitation to emit a green photon. The full light emission profiles of probes **β-gal-MA-Tetrox** and **β-gal-MA-AD** in the presence of a high concentration of β-gal are presented in Figure 3B1. As expected, probe **β-gal-MA-Tetrox** generated a fast chemiluminescent profile with an intense initial light emission signal that completely decayed within less than 50 min. On the other hand, the light emission profile of probe **β-gal-MA-AD**, is increased slowly, to reach a maximum signal after 90 min and then decayed for another 160 min.

The signal-to-noise (S/N) ratio obtained by probe **β-gal-MA-Tetrox** for the total light emission profile is about 50-fold higher than that of probe **β-gal-MA-AD** (Figure 3B2). The S/N ratios of the two probes were then measured under saturation kinetics conditions with low enzyme concentration (Figure 3B3). Under such conditions, the S/N value observed for probe **β-gal-MA-Tetrox** after 14 min was about 47-fold higher than that of probe **β-gal-MA-AD**. The superior S/N of **β-gal-MA-Tetrox** is achieved mainly because this probe is hydrolytically more stable than probe **β-gal-MA-AD** and thus has a lower background signal.
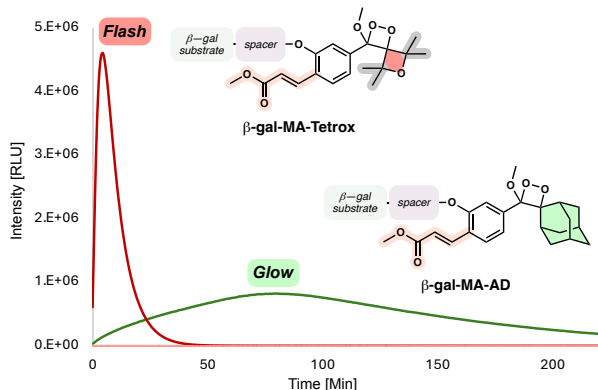
Next, a comparative evaluation of the LOD (limit-of-detection) values of the two probes for the detection of β-gal activity was conducted (Figure 3B4). In correlation with the results of the saturation kinetics, probe **β-gal-MA-Tetrox** presented superior detection sensitivity with a 43-fold lower LOD value compared to the adamantyl analog. These results demonstrate the significant increase in detection sensitivity achieved by the spiro-dioxetane fused to a tetramethyl-oxetanyl unit.
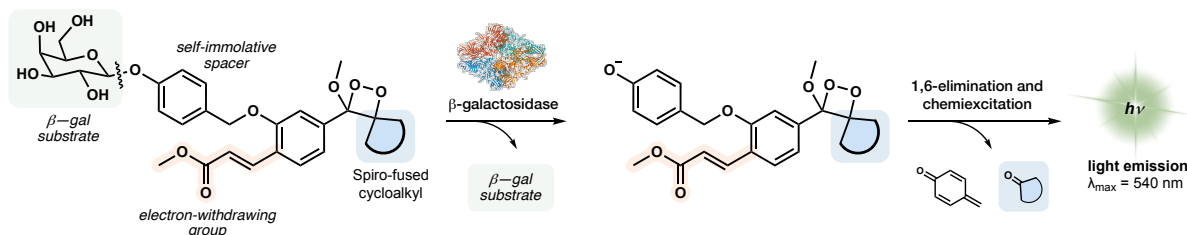
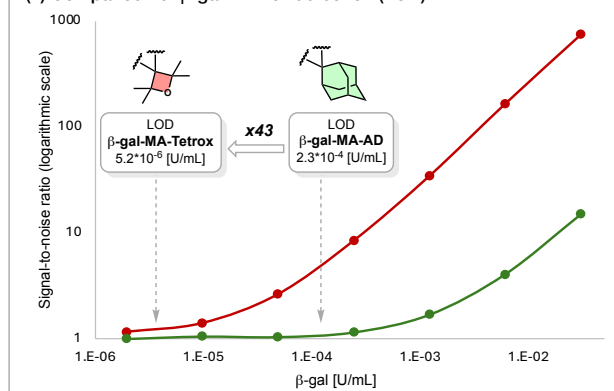**Figure 3**. (A) Chemiluminescent activation pathway of Methyl-Acrylate 1,2-dioxetanes bearing a β-gal-responsive trigger. (B) Comparison between probes **β-gal-MA-Tetrox** and **β-gal-MA-AD**. 1. Full chemiluminescent kinetic profiles. 2. Signal-to-noise ratio of a full light emission profile (The signal-to-noise value is the ratio between the total emitted light in the presence or absence of β-gal). 3. Signal-to-noise ratio of total light emission kinetic profile under saturation kinetic conditions [0.003 U/mL of β-gal]. 4. β-gal Limit-of-detection. For additional information see supporting Figures S5–S11; for detailed procedures see the experimental protocols section of the supporting information.

The exceptional detection sensitivity of probe **β-gal-MA-Tetrox** towards β-gal activity prompted us to evaluate its performance in live bacterial assays (Figure 4). Cell assays were performed directly in Lysogeny broth (LB) bacterial growth medium to ensure a precise assessment of bacterial enzymatic activity, without the need for media replacement with an assay buffer prior to measurements. This method of

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measurement preserves enzymes that might otherwise be washed away. We initially sought to assess the effect of substituting the oxetane ring with four methyl groups, on the stability of probe **β-gal-MA-Tetrox** in cell-growth media. As a control, we used the non-substituted cyclobutyl-dioxetane probe **β-gal-MA-CB** (Figure 4A). As expected, the non-substituted probe **β-gal-MA-CB** exhibited extremely low chemical stability undergoing rapid decomposition ($t_{1/2}$ = 2 min) in the cell media. However, the sterically hindered dioxetane probe **β-gal-MA-Tetrox** showed substantially higher stability with a $t_{1/2}$ = 92 min, which is nearly 50-fold superior to that of probe **β-gal-MA-CB** (Figure 4A and supporting Figure S12). While the enhanced stability attained for the probe **β-gal-MA-Tetrox** in cell media remains below that of the adamantyl probe **β-gal-MA-AD**, a half-life of 92 min is more than sufficient for cell assay measurements.

Probes **β-gal-MA-Tetrox**, **β-gal-MA-CB**, and **β-gal-MA-AD** were then incubated in the presence of *Escherichia coli* (ATCC 9637) in LB medium, and the light emission signal was monitored over 15 min. As anticipated by the stability data, probe **β-gal-MA-CB** did not show any light emission due to its immediate decomposition. Remarkably, due to its enhanced stability and fast chemiexcitation mode, probe **β-gal-MA-Tetrox** exhibited a noticeable S/N ratio of 9.2 and the capability to detect β-gal activity with high statistical significance within less than one minute since the measurement was initiated. Under the same conditions, after 15 minutes, probe **β-gal-MA-AD** achieved a low S/N value of 1.2 as a result of its slow rate of chemiexcitation. Based on these findings, probe **β-gal-MA-Tetrox** presented a 64-fold improvement in LOD value compared to probe **β-gal-MA-AD**, enabling the detection of the minimal number of $4.7*10^5$ bacterial cells directly in LB cell growth medium(Figure 4C). These results effectively demonstrate the superior detection sensitivity of a chemiluminescent dioxetane probe with a flash mode of chemiexcitation over one with a glow chemiexcitation mode.
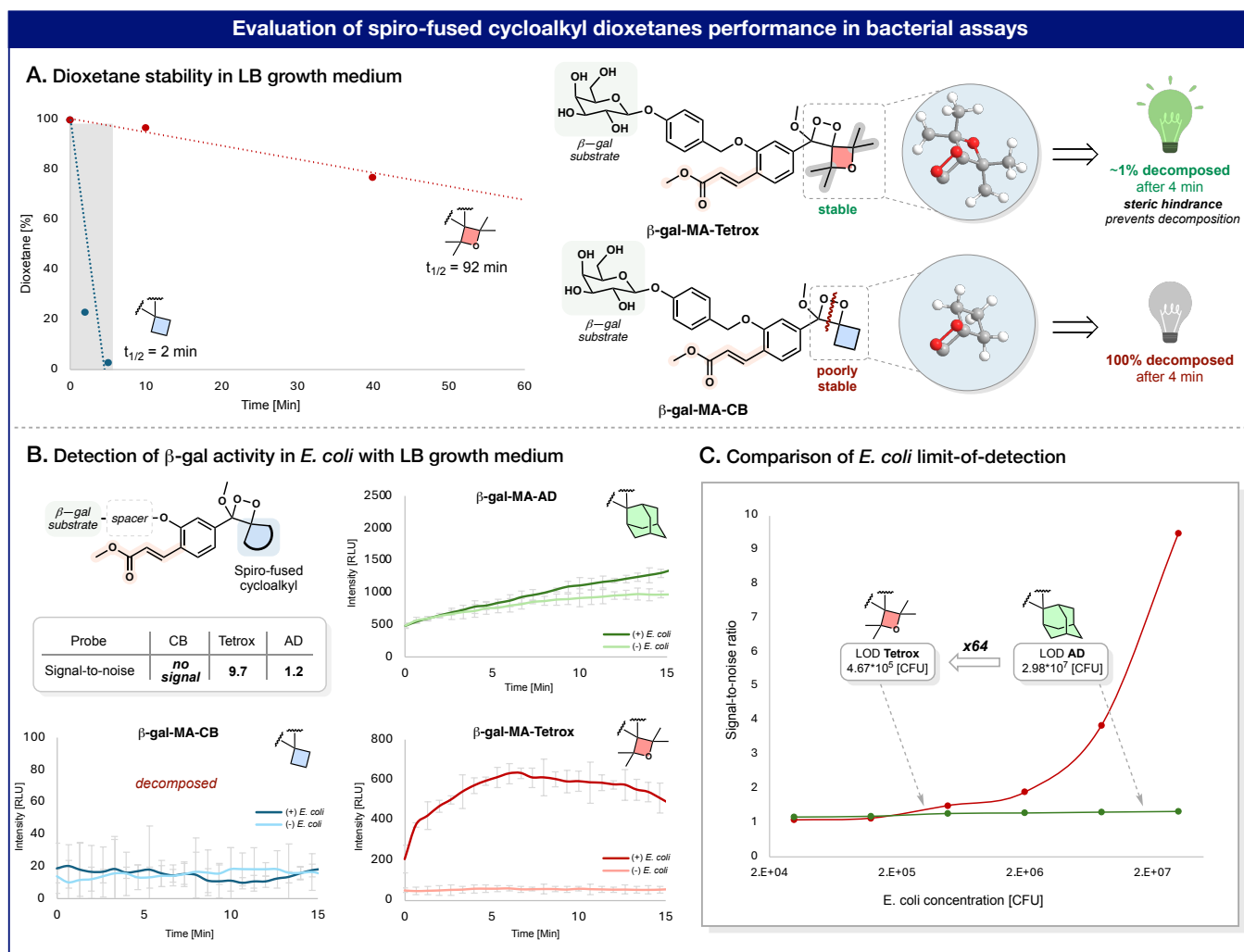
Figure 4. (A) Stability of the probes **β-gal-MA-Tetrox** and **β-gal-MA-CB** in LB. (B) Chemiluminescent kinetic profiles and signal-to-noise ratios in the presence or absence of *E. coli* in LB bacterial growth medium. (C) Determination of *E. coli* limit of detection (Probe **β-gal-MA-CB** was not included in the LOD measurement since it immediately decomposes in LB and therefore could not detect any β-gal activity). For additional information see supporting Figures S12–S16; for detailed procedures see the Biology: General Information and Procedures section of the supporting information.

The successful performance of probe **β-gal-MA-Tetrox** in generating flash chemiluminescence and detecting bacterial enzymatic activity has motivated us to extend our investigation to assess the capability of an analogous probe in detecting enzymatic activity produced by mammalian cells. Leucine aminopeptidase (LAP) is a protease known for selectively cleaving *N*-terminal leucine residues from a diverse range of substrates. Given its association with various diseases and pathological conditions, aberrant levels of LAP often serve as diagnostic or prognostic biomarkers.[25, 26] Previous studies have demonstrated that chemiluminescent dioxetane probes can detect LAP overexpression in certain cancer cells compared to their healthy counterparts.[27, 28]

We next sought to compare the detection sensitivity of a LAP spiro-dioxetane probe fused with a tetramethyl-oxetane unit to that of spiro-adamantyl dioxetane probe (Figure 5). Therefore, two new probes aimed for the detection of LAP activity were synthesized. The molecular structures of probe **Leu-AN-Tetrox** and probe **Leu-AN-AD** are shown in Figure 5A. These probes include an *ortho*-acrylonitrile substituent, which facilitates a higher chemiluminescent quantum yield (about 4-fold) compared to the *ortho*-metylacrylate group (Supporting figures S17-S18). Ovarian cancer cells (OVCAR-3) were chosen for the comparison based on previous reports of LAP overexpression in this cell line.[25] The incubation of probe **Leu-AN-Tetrox** and **Leu-AN-AD** with OVCAR-3 cells for 15 minutes resulted in a statistically significant response for probe **Leu-AN-Tetrox** (S/N value of 2.6), while no significant response was observed for probe **Leu-AN-AD** (Figure 5B). Pre-incubation of the cells with the known LAP inhibitor Bestatin (also known by its chemical name Ubenimex) for 30 minutes resulted in complete inhibition of the light emission signal produced by probe **Leu-AN-Tetrox**.

Taking advantage of probe **Leu-AN-Tetrox** enhanced detection sensitivity, a screen of LAP activity across three types of cancer cells (Ovarian, Colon, and Lung) and one normal cell line (Fibroblasts) was conducted. Probe **Leu-AN-Tetrox** produced a signal with a statistically significant increase for LAP activity in all four cell lines, which was completely inhibited by the addition of the LAP inhibitor Bestatin (Figure 5C and supporting Figure S20). Although probe **Leu-AN-Tetrox** exhibited detection of LAP activity for all four cell lines, the S/N ratio values obtained by the ovarian cancer and the colon cancer cell lines (OVCAR-3 and HCT-116) were slightly elevated compared to the lung cancer and the normal cell lines. Since most cell lines produced some native level of LAP, the high performance of probe **Leu-AN-Tetrox** could detect even basal levels of LAP activity. These results are consistent with previously reported LAP overexpression data for these cancer cell lines (ovarian and colon). Other known optical probes based on fluorescence, could not achieve detection of basal LAP activity in normal cells like fibroblasts due to their insufficient sensitivity.

The obtained results indicate that LAP might serve as a biomarker for ovarian and colon cancer due to its overexpression in these cell lines. However, the similar S/N ratio obtained by probe **Leu-AN-Tetrox** for LAP activity in lung cancer cells compared to the fibroblast cells suggests that LAP should not be utilized as a biomarker for this type of cancer, unless a comprehensive investigation of LAP activity across a large number of cell lines demonstrates statistical significance.
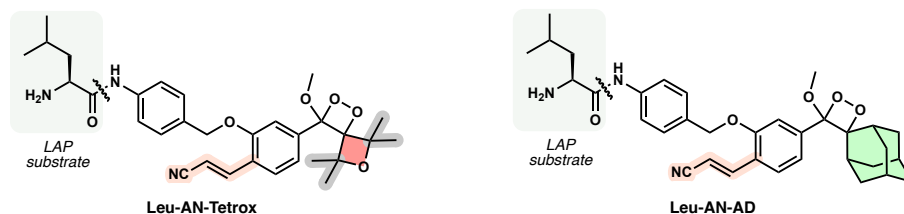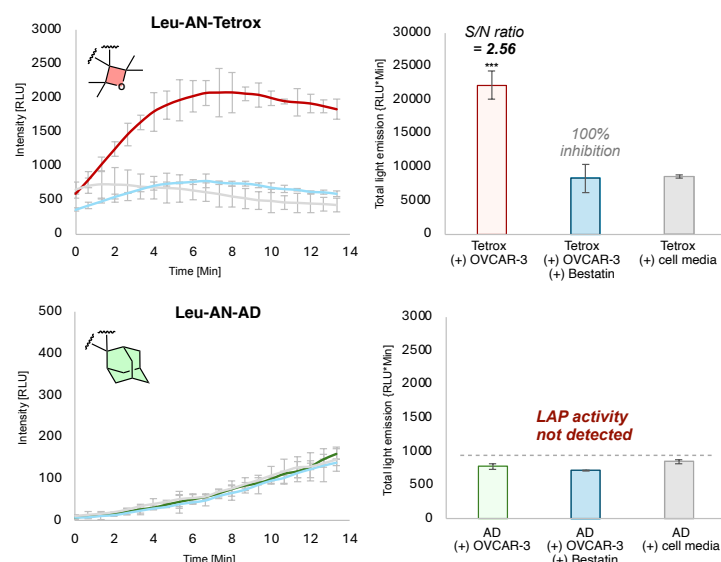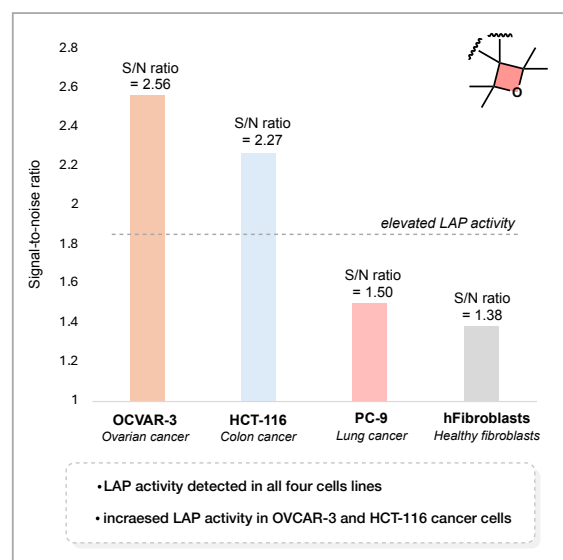
Figure 5. (A) Molecular structure of the chemiluminescent probes for the detection of LAP activity in mammalian cells. (B) Chemiluminescent kinetic profiles (top) and total light emission (bottom) of probes **Leu-AN-Tetrox** and **Leu-AN-AD** with either medium only, OVCAR-3 cells alone, or OVCAR-3 cells preincubated with the LAP inhibitor Bestatin [50μM] for 30 min. (C) Probes **Leu-AN-Tetrox** signal-to-noise ratios in the presence of three cancer cell lines (OVCAR-3, HCT-116, and PC-9) and one control cell line (hFibroblasts).

chemiluminescent assays are among the most sensitive diagnostic methods for the detection of enzymatic and bioanalytes activities due to their exceptionally high signal-to-noise ratio. The chemiluminescent signal generated by these probes can be classified as either flash-type or glow-type chemiluminescence, with each type having its advantages and disadvantages depending on the specific application. Generally, assays that require enhanced sensitivity achieve superior results with flash-type probes, as they generate a strong light emission that is directly correlated with a high signal-to-noise ratio.

The discovery of the spiro-strained-cyclobutyl dioxetanes yielded chemiluminescent probes with unprecedented detection sensitivity due to their extremely fast chemiexcitation flash mode. However, the chemical stability of these dioxetanes was severely compromised. While their thermal stability is

sufficient for prolonged storage at low temperatures and several hours of usage at room temperature, the inferior chemical stability restricts their application to assays conducted under conditions free of any reducing agents. For example, these probes can be effectively employed in enzymatic assays conducted in a standard buffer solution, such as the detection of β-gal activity in PBS 7.4. However, since certain enzymes require a working buffer that includes a reducing agent such as glutathione, in which the decomposition of spiro-cyclobutyl dioxetane occurs immediately, the application of these probes for the detection of such enzymes is not possible. Consequently, the utilization of spiro-cyclobutyl dioxetane probes in a complex biological system, such as cells or tissues, is impractical.

Applying steric hindrance in the vicinity of the dioxetane along with spiro-strain and an electronegative heteroatom, led to a luminophore with enhanced stability and a slightly accelerated chemiexcitation rate compared to the unsubstituted spiro-cyclobutyl (As evident from Figure 2B and Figure 4A). Turn-ON probes comprised of phenoxy-1,2-dioxetane chemiluminophore with a tetramethyl-oxetanyl unit, instead of an adamantyl unit, exhibited a substantial enhancement in detection sensitivity, with an observed increase ranging from 43 to 77-fold. The enhanced sensitivity was demonstrated with an *ortho*-methyl acrylate dioxetane, which was employed for the detection of β-gal activity using directly the enzyme or live bacteria (Figures 3-4), and with an *ortho*-acrylonitrile dioxetane which was employed for the detection of LAP activity in cancer cells and in bacteria (Figure 5 and supporting Figures S21-S22). The flash chemiexcitation in combination with elevated chemical stability achieved through the incorporation of a tetramethyl-oxetanyl unit presents a fascinating opportunity to explore new applications of flash-type chemiluminescence in complex biological contexts such as cell-based drug screening platforms and *in-vivo* imaging.

In summary, we have developed a new phenoxy-spiro-1,2-dioxetane luminophore fused to a distinct strained molecular function composed of a tetramethyl-oxetane motif. The chemiexcitation rate of the dioxetane luminophore was significantly accelerated through a spiro-strain-release effect, generated by the tetramethyl-oxetane motif. Remarkably, chemiluminescent luminophore based on the spiro-dioxetane containing the tetramethyl-oxetanyl unit exhibited a chemiexcitation rate that was 128-fold faster, when compared to the classic spiro-adamantyl-dioxetane. Turn-on probes composed of tetramethyl-oxetanyl spiro-dioxetane exhibited significantly improved chemical stability in bacterial and mammalian cell media in comparison to previously developed dioxetane probes fused to a cyclobutyl unit. The superior chemical stability enables the tetramethyl-oxetanyl dioxetane probe to detect β-gal activity

with enhanced sensitivity in *E. coli* bacterial assays and Leucine aminopeptidase activity in tumoral cell lines. Overall, the results obtained in this study assist in advancing the design of more effective phenoxy-1,2-dioxetane chemiluminescent probes and underscore the significance of strain-release techniques for enhancing the detection sensitivity of chemiluminescence assays.

References

(1) Roda, A.; Guardigli, M. Analytical chemiluminescence and bioluminescence: latest achievements and new horizons. *Anal. Bioanal. Chem.* **2012**, *402* (1), 69-76. DOI: 10.1007/s00216-011-5455-8.

(2) Gnaim, S.; Green, O.; Shabat, D. The emergence of aqueous chemiluminescence: new promising class of phenoxy 1,2-dioxetane luminophores. *Chem. Commun.* **2018**, *54* (17), 2073-2085. DOI: 10.1039/c8cc00428e.

(3) Blau, R.; Shelef, O.; Shabat, D.; Satchi-Fainaro, R. Chemiluminescent probes in cancer biology. *Nat. Rev. Bioeng.* **2023**, *1* (9), 648–664. DOI: 10.1038/s44222-023-00074-0.

(4) Dodeigne, C.; Thunus, L.; Lejeune, R. Chemiluminescence as a diagnostic tool. A review. *Talanta* **2000**, *51* (3), 415-439. DOI: 10.1016/S0039-9140(99)00294-5.

(5) Schaap, A. P.; Chen, T. S.; Handley, R. S.; Desilva, R.; Giri, B. P. Chemical and Enzymatic Triggering of 1,2-Dioxetanes .2. Fluoride-Induced Chemiluminescence from Tert-Butyldimethylsilyloxy-Substituted Dioxetanes. *Tetrahedron Lett.* **1987**, *28* (11), 1155-1158. DOI: Doi 10.1016/S0040-4039(00)95313-9.

(6) Lampinen, J. R., M.; Perälä, A.; Kytöniemi, V.; Harinen, R. . *Comparison of Flash and Glow ATP Assays with Thermo Scientific Varioskan Flash Luminometry*. Application Note: AP-MIB-ARIO12–0108. https://assets.thermofisher.com/TFS-Assets/LCD/Application-Notes/D10338~.pdf (accessed 2024-05-04).

(7) Tannous, R.; Shelef, O.; Gutkin, S.; David, M.; Leirikh, T.; Ge, L.; Jaber, Q.; Zhou, Q.; Ma, P.; Fridman, M.; et al. Spirostrain-Accelerated Chemiexcitation of Dioxetanes Yields Unprecedented Detection Sensitivity in Chemiluminescence Bioassays. *ACS Cent. Sci.* **2024**, *10* (1), 28-42. DOI: 10.1021/acscentsci.3c01141.

(8) Haris, U.; Lippert, A. R. Exploring the Structural Space of Chemiluminescent 1,2-Dioxetanes. *ACS Sens.* **2022**, *8* (1), 3-11. DOI: 10.1021/acssensors.2c02371.

(9) Matsumoto, M.; Kobayashi, H.; Matsubara, J.; Watanabe, N.; Yamashita, S.; Oguma, D.; Kitano, Y.; Ikawa, H. Effect of allylic oxygen on the reaction pathways of singlet oxygenation: Selective formation of 1,2-dioxetanes from 1-alkoxymethyl-2-aryl-1-tert-butyl-2-methoxyethylenes. *Tetrahedron Lett.* **1996**, *37* (3), 397-400. DOI: 10.1016/0040-4039(95)02185-X.

(10) Green, O.; Eilon, T.; Hananya, N.; Gutkin, S.; Bauer, C. R.; Shabat, D. Opening a Gateway for Chemiluminescence Cell Imaging: Distinctive Methodology for Design of Bright Chemiluminescent Dioxetane Probes. *ACS Cent. Sci.* **2017**, *3* (4), 349-358. DOI: 10.1021/acscentsci.7b00058.

(11) Tannous, R.; Shelef, O.; Kopp, T.; Fridman, M.; Shabat, D. Hyper-Responsive Chemiluminescent Probe Reveals Distinct PYRase Activity in Pseudomonas aeruginosa. *Bioconjug. Chem.* **2024**, *35* (4), 472-479. DOI: 10.1021/acs.bioconjchem.4c00015.

(12) Shelef, O.; Kopp, T.; Tannous, R.; Arutkin, M.; Jospe-Kaufman, M.; Reuveni, S.; Shabat, D.; Fridman, M. Enzymatic Activity Profiling Using an Ultrasensitive Array of Chemiluminescent Probes for Bacterial Classification and Characterization. *J. Am. Chem. Soc.* **2024**, *146* (8), 5263-5273. DOI: 10.1021/jacs.3c11790.

(13) Gutkin, S.; Tannous, R.; Jaber, Q.; Fridman, M.; Shabat, D. Chemiluminescent duplex analysis using phenoxy-1,2-dioxetane luminophores with color modulation. *Chem. Sci.* **2023**, *14* (25), 6953-6962. DOI: 10.1039/d3sc02386a.

(14) Shelef, O.; Gutkin, S.; Feder, D.; Ben-Bassat, A.; Mandelboim, M.; Haitin, Y.; Ben-Tal, N.; Bacharach, E.; Shabat, D. Ultrasensitive chemiluminescent neuraminidase probe for rapid screening and identification of small-molecules with antiviral activity against influenza A virus in mammalian cells. *Chem. Sci.* **2022**, *13* (42), 12348-12357. DOI: 10.1039/d2sc03460c.

(15) Shelef, O.; Sedgwick, A. C.; Pozzi, S.; Green, O.; Satchi-Fainaro, R.; Shabat, D.; Sessler, J. L. Turn on chemiluminescence-based probes for monitoring tyrosinase activity in conjunction with biological thiols. *Chem. Commun.* **2021**, *57* (86), 11386-11389. DOI: 10.1039/d1cc05217a.

(16) Babin, B. M.; Fernandez-Cuervo, G.; Sheng, J.; Green, O.; Ordonez, A. A.; Turner, M. L.; Keller, L. J.; Jain, S. K.; Shabat, D.; Bogyo, M. Chemiluminescent Protease Probe for Rapid, Sensitive, and Inexpensive Detection of Live Mycobacterium tuberculosis. *ACS Cent. Sci.* **2021**, *7* (5), 803-814. DOI: 10.1021/acscentsci.0c01345.

(17) Gutkin, S.; Green, O.; Raviv, G.; Shabat, D.; Portnoy, O. Powerful Chemiluminescence Probe for Rapid Detection of Prostate Specific Antigen Proteolytic Activity: Forensic Identification of Human Semen. *Bioconjugate Chem.* **2020**, *31* (11), 2488-2493. DOI: 10.1021/acs.bioconjchem.0c00500.

(18) Roth-Konforti, M.; Green, O.; Hupfeld, M.; Fieseler, L.; Heinrich, N.; Ihssen, J.; Vorberg, R.; Wick, L.; Spitz, U.; Shabat, D. Ultrasensitive Detection of Salmonella and Listeria monocytogenes by Small-Molecule Chemiluminescence Probes. *Angew. Chem. Int. Ed.* **2019**, *58* (30), 10361-10367. DOI: 10.1002/anie.201904719.

(19) Hananya, N.; Green, O.; Blau, R.; Satchi-Fainaro, R.; Shabat, D. A Highly Efficient Chemiluminescence Probe for the Detection of Singlet Oxygen in Living Cells. *Angew. Chem. Int. Ed.* **2017**, *56* (39), 11793-11796. DOI: 10.1002/anie.201705803.

(20) Green, O.; Gnaim, S.; Blau, R.; Eldar-Boock, A.; Satchi-Fainaro, R.; Shabat, D. Near-Infrared Dioxetane Luminophores with Direct Chemiluminescence Emission Mode. *J. Am. Chem. Soc.* **2017**, *139* (37), 13243-13248. DOI: 10.1021/jacs.7b08446.

(21) Huang, J.; Jiang, Y.; Li, J.; Huang, J.; Pu, K. Molecular Chemiluminescent Probes with a Very Long Near-Infrared Emission Wavelength for in Vivo Imaging. *Angew. Chem. Int. Ed.* **2021**, *60* (8), 3999-4003. DOI: 10.1002/anie.202013531.

(22) Huang, J.; Huang, J.; Cheng, P.; Jiang, Y.; Pu, K. Near-Infrared Chemiluminescent Reporters for In Vivo Imaging of Reactive Oxygen and Nitrogen Species in Kidneys. *Adv. Funct. Mater.* **2020**, *30* (39), 2003628. DOI: 10.1002/adfm.202003628.

(23) Kagalwala, H. N.; Gerberich, J.; Smith, C. J.; Mason, R. P.; Lippert, A. R. Chemiluminescent 1,2-Dioxetane Iridium Complexes for Near-Infrared Oxygen Sensing. *Angew. Chem. Int. Ed.* **2022**, *61* (12), e202115704. DOI: 10.1002/anie.202115704.

(24) Son, S.; Won, M.; Green, O.; Hananya, N.; Sharma, A.; Jeon, Y.; Kwak, J. H.; Sessler, J. L.; Shabat, D.; Kim, J. S. Chemiluminescent Probe for the In Vitro and In Vivo Imaging of Cancers Over-Expressing NQO1. *Angew. Chem. Int. Ed.* **2019**, *58* (6), 1739-1743. DOI: 10.1002/anie.201813032 From NLM Medline.

(25) Li, Z. J.; Wang, C. Y.; Xu, L.; Zhang, Z. Y.; Tang, Y. H.; Qin, T. Y.; Wang, Y. L. Recent Progress of Activity-Based Fluorescent Probes for Imaging Leucine Aminopeptidase. *Biosensors* **2023**, *13* (7), 752. DOI: 10.3390/bios13070752.

(26) Scott, J. I.; Deng, Q.; Vendrell, M. Near-Infrared Fluorescent Probes for the Detection of Cancer-Associated Proteases. *ACS Chem. Biol.* **2021**, *16* (8), 1304-1317. DOI: 10.1021/acschembio.1c00223.

(27) Gunduz, H.; Acari, A.; Cetin, S.; Almammadov, T.; Pinarbasi-Degirmenci, N.; Dirak, M.; Cingoz, A.; Kilic, E.; Bagci-Onder, T.; Kolemen, S. Directing chemiluminescent dioxetanes to mitochondria: A cationic luminophore enables in vitro and in vivo detection of cancer cells upon enzymatic activation. *Sensor. Actuat. B: Chem.* **2023**, *383*, 133574. DOI: 10.1016/j.snb.2023.133574.

(28) Wang, B.; Chen, Z.; Cen, X.; Liang, Y.; Tan, L.; Liang, E.; Zheng, L.; Zheng, Y.; Zhan, Z.; Cheng, K. A highly selective and sensitive chemiluminescent probe for leucine aminopeptidase detection in vitro, in vivo and in human liver cancer tissue. *Chem. Sci.* **2022**, *13* (8), 2324-2330. DOI: 10.1039/d1sc06528a.