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A microtubule-localizing activity-based sensing fluorescent probe for imaging hydrogen peroxide in living cells

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ABSTRACT

Hydrogen peroxide (H_2O_2) is a major reactive oxygen species (ROS) in living systems with broad roles spanning both oxidative stress and redox signaling. Indeed, owing to its potent redox activity, regulating local sites of H_2O_2 generation and trafficking is critical to determining downstream physiological and/or pathological consequences. We now report the design, synthesis, and biological evaluation of Microtubule Peroxy Yellow 1 (MT-PY1), an activity-based sensing fluorescent probe bearing a microtubule-targeting moiety for detection of H_2O_2 in living cells. MT-PY1 utilizes a boronate trigger to show a selective and robust turn-on response to H_2O_2 in aqueous solution and in living cells. Live-cell microscopy experiments establish that the probe co-localizes with microtubules and retains its localization after responding to changes in levels of H_2O_2, including detection of endogenous H_2O_2 fluxes produced upon growth factor stimulation. This work adds to the arsenal of activity-based sensing probes for biological analytes that enable selective molecular imaging with subcellular resolution.

Main text

Hydrogen peroxide (H_2O_2) is a central member of the reactive oxygen species (ROS) family and is continually produced by foundational cellular processes that span respiration, oxidase catalysis, protein folding, and peroxisome activity[1,2]. On the other hand, dysregulation of H_2O_2 triggers oxidative stress and damage cascades that are implicated in aging[3] and disease states, including cancer[4,5], inflammation[6,7], diabetes[8], and neurodegeneration[9]. In the context of H_2O_2 as a physiological signal[10,11], controlled and localized generation of this ROS occurs in response to various stimuli such as growth factors, cytokines, and neurotransmitters[12–15], where membrane-bound NADPH oxidases play a pivotal role in generating H_2O_2 fluxes in confined cellular spaces to react with downstream targets[16–18].

To meet the need for identifying and characterizing the diverse sources and functions of H_2O_2 as a transient redox messenger, we[19–21] and others[22–27] have developed molecular probes for selective monitoring of H_2O_2 to selectively disentangle its contributions from other ROS. In particular, our laboratory has advanced the use of H_2O_2-mediated boronate oxidation for H_2O_2 detection[19,21] as part of a larger program in activity-based sensing for selective monitoring of biological analytes[28,29]. Since our initial report of Peroxyfluor-1 (PF1) that established selectivity for H_2O_2 detection over competing ROS and use in living cells[30], we have designed activity-based boronate fluorescent probes for monitoring H_2O_2 with varying excitation/emission colors[31], reagents with increased sensitivity for visualizing endogenous H_2O_2 at signaling levels[32,33], two-color dyes for ratiometric H_2O_2 detection[34,35], bifunctional probes for organelle-specific H_2O_2 detection[36,37], cell-trappable sensors for intracellular H_2O_2 signaling and identification of peroxide channels and peroxide-dependent neurogenesis[38,39], and more recently tandem activity-based sensing/labeling for identifying trans-cellular H_2O_2 signaling in microglia-neuron co-cultures[40]. Beyond acting as a general H_2O_2 caging group for fluorophores, the versatility of boronate triggers for H_2O_2 detection also enables development of the Peroxy Caged Luciferin family of bioluminescent H_2O_2 reporters based on caged luciferins[41,42], histochemical analysis with Peroxymycin-1, a puromycin-based H_2O_2 detection reagent[43], and a caged radiotracer for positron emission tomography (PET) imaging of H_2O_2[44].

Against this backdrop, a key challenge to studying H_2O_2 signaling is the transient and localized nature of H_2O_2 fluxes. Indeed, traditional boronate fluorescent probes are diffusible before and after analyte detection, which can limit spatial resolution in monitoring localized H_2O_2 fluxes. To address this issue, we have reported SNAP Peroxy Green 1 (SNAP-PG1) and Peroxy Green 1 Fluoromethyl (PG1-FM) reagents that

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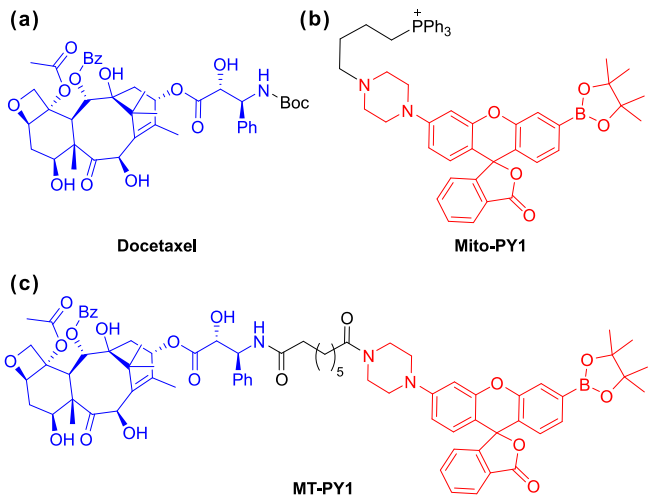
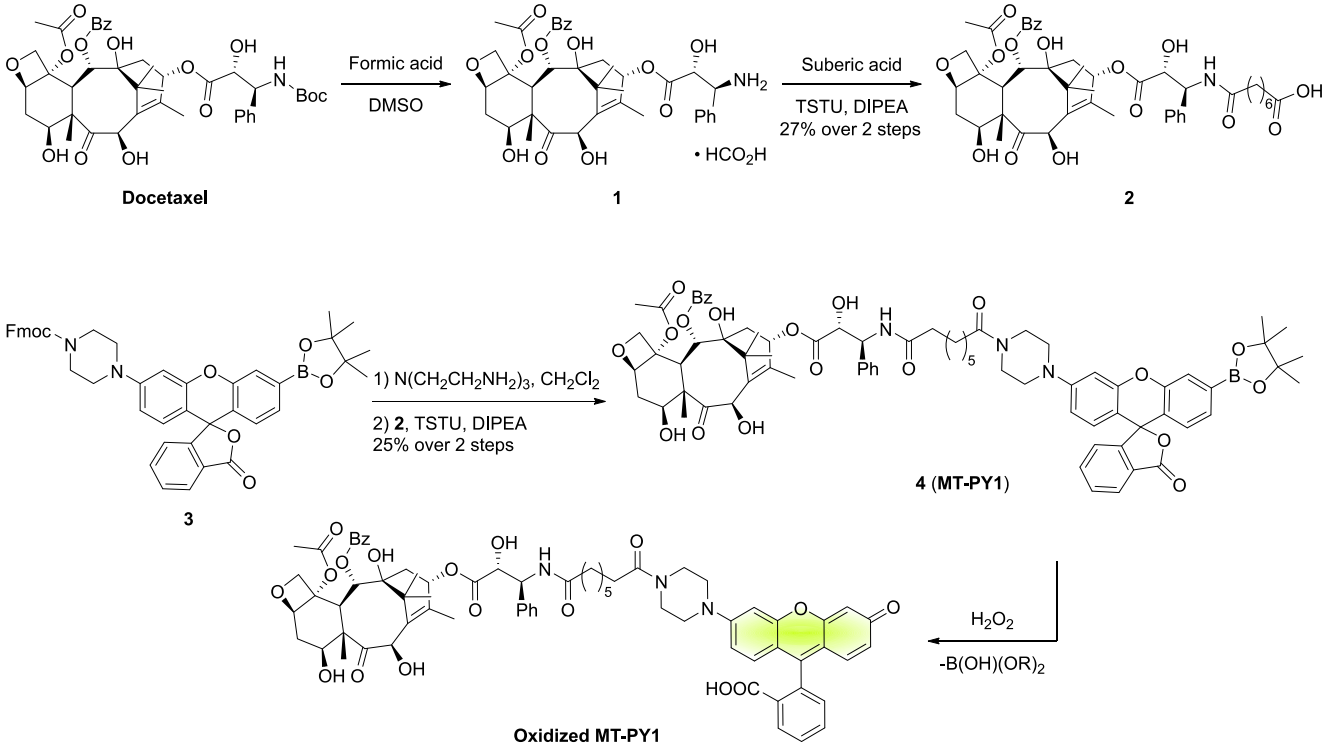


Figure 1. Structures of (a) microtubule-targeting molecule docetaxel; (b) mitochondria-localizing H_2O_2 probe Mito-PY1, and (c) microtubule-localizing H_2O_2 probe MT-PY1.

covalently label H_2O_2-responsive dyes onto intracellular proteins to limit probe diffusion[37,40]. Along these lines, we now report the design, synthesis, and evaluation of Microtubule Peroxy Yellow 1 (MT-PY1), a unique H_2O_2-responsive probe that localizes to microtubules that form part of the cytoskeleton and can retain its spatial localization before and after reporting on changes in H_2O_2 levels.

Inspired by reports that conjugate fluorophores to taxoids as a microtubule-targeting group for conventional[45,46] and super-resolution[47–49] imaging of the cytoskeleton in living cells, along with leveraging our laboratory's previous work on a modular rhodol scaffold in the development of a mitochondrial-targeted H_2O_2 probe, Mito-PY1[36], we designed and synthesized MT-PY1 by linking a boronate rhodol to the primary amine of a Boc-deprotected docetaxel via a suberic acid linker (Figure 1a). The analogous H_2O_2 probe without a targeting moiety, Peroxy Yellow 1 (PY1), has already been reported in a previous paper from our laboratory (Supplementary Figure S1)[33]. The synthesis of MT-PY1 is depicted in Scheme 1. The Boc-group on docetaxel was removed by treatment with formic acid, and the dicarboxylic linker was introduced with TSTU coupling to afford **2**. The Fmoc-protected H_2O_2 sensing motif **3** shared the same initial steps with Mito-PY1. However, the amide formation step with TSTU was incompatible in the presence of dibenzofulvene as a byproduct of Fmoc-deprotection. This byproduct was removed by using tris(2-aminoethyl)amine (TAEA) as both deprotection reagent and dibenzofulvene scavenger[50], which enables facile purification of the deprotected secondary amine for coupling with **3** to afford MT-PY1.

With the MT-PY1 probe in hand, we first evaluated its fluorescence turn-on behavior for activity-based sensing of H_2O_2 in *in vitro* experiments. We incubated the probe at 37 °C in aqueous media buffered to neutral pH for 1 h and observed a negligible measurable fluorescence response. In contrast, after incubation with 100 μM H_2O_2, we observed a 12-fold turn-on response within 1 h (Figure 2a), which is comparable with previously published boronate probes for activity-based sensing of H_2O_2. This result suggests that the introduction of docetaxel as the localizing moiety does not interfere with the activity-based sensing reaction between H_2O_2 and boronic ester. We further tested the response of MT-PY1 to other biologically relevant reactive oxygen species (ROS) and nitrogen species (RNS) (Figure 2b). The results show that MT-PY1 reacts primarily with H_2O_2, which is in agreement with its non-targeted analog PY1 and other previously reported boronate-based H_2O_2 probes. The only exception is peroxynitrite ($ONOO^-$), which is not surprising given that some boronic esters also react with this RNS[51,52]. Indeed, we note that despite the fact that the relative in vitro reactivity of boronates with peroxynitrite can be comparable or faster than with hydrogen peroxide in aqueous buffer, in biological contexts one must consider that $ONOO^-$ is a highly reactive species that exists in cells with exceedingly short lifetimes of 10–20 ms and estimated concentrations in the sub-nanomolar range[53], whereas local H_2O_2 concentrations can approach micromolar levels[54]. As such, we advocate for the use of a straightforward control experiment in cells and other biological



Scheme 1. Synthesis of MT-PY1 and its fluorescence turn-on reaction upon activity-based sensing of H_2O_2.

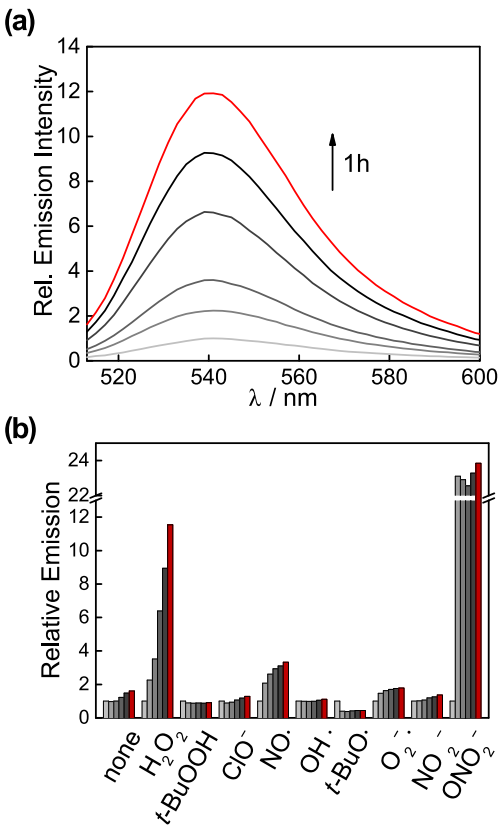


Figure 2. *In vitro* characterization of MT-PY1. (a) Fluorescence emission spectrum of 5 μM MT-PY1 in 25 mM HEPES buffer pH 7.4 (bottom), and its turn-on response after treatment with 100 μM H_2O_2 at 37 °C for 5, 15, 30, 45 and 60 min. (b) Fluorescence responses of 5 μM MT-PY1 to 100 μM of various reactive oxygen and nitrogen species at 37 °C after 5, 15, 30, 45 and 60 min of incubation.

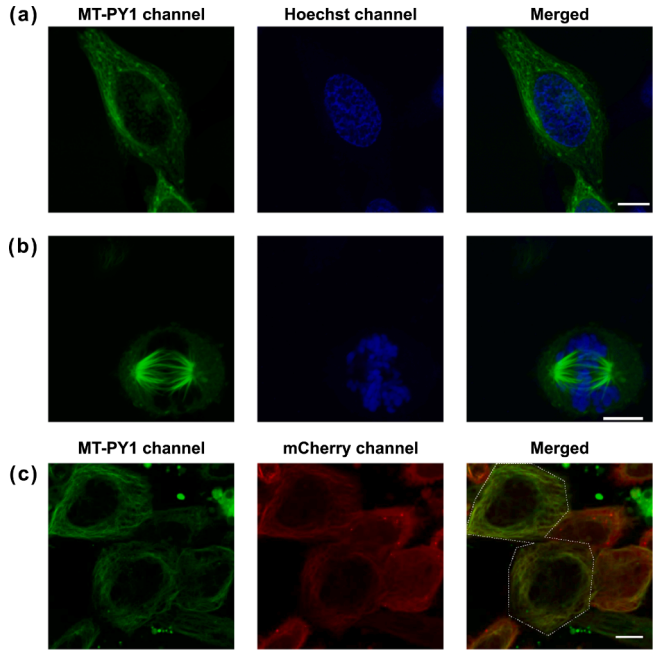


Figure 3. Confocal microscopy images of MT-PY1 localization in HeLa cells. Cells were incubated with 1 μM MT-PY1 for 15 min at 37 °C prior to imaging. (a) A HeLa cell in metaphase. (b) A HeLa cell in interphase. Cell nucleus is stained by Hoechst 33342 in (a) and (b). (c) HeLa cells expressing mCherry-tubulin stained with MT-PY1. Enlarged image is shown in Supplementary Figure S2. Scale-bar: 10 μm.

models with a nitric oxide synthase (NOS) inhibitor. This type of experiment can distinguish between peroxide-dependent and peroxynitrite-dependent signals using boronate reagents, as the former will be insensitive to NOS inhibition whereas the latter will be sensitive to blocking of an NO source.

We next evaluated the MT-PY1 reagent in cell imaging experiments by first testing whether the probe is able to localize onto microtubule structures. As expected, MT-PY1 showed an even distribution in the cytosol, visualizing a network of filaments that resembles the cytoskeleton outside the nucleus (Figure 3a). Moreover, images of a dividing cell also displayed a structure with the shape of a spindle apparatus with chromosomes in the center (Figure 3b). To confirm the subcellular localization of the MT-PY1 probe, we performed dual-color imaging experiments with cells that were transfected with mCherry-tubulin that assemble to form mCherry-decorated microtubules. Images of these cells after staining with MT-PY1 are shown in Figure 3c. Since taxoids show limited binding to free tubulin and only associate with assembled microtubules, cells in the MT-PY1 channel show a well-defined filament structure. In contrast, due to the different expression levels of mCherry-tubulin, some cells showed free, unassembled tubulin which light up the entire cytoplasm with less pronounced filament features. For cells that show primarily microtubule mCherry signal (outlined by dotted lines in Figure 3c), we observed strong colocalization between MT-PY1 in the green channel and mCherry-labeled microtubule in the red channel with a Pearson correlation coefficient of 0.92. Moreover, the probe is not cytotoxic at the low doses needed for imaging, with no observable inhibition effect even at 4 μM over 24 h incubation, which is well above the typical dose/time for an imaging experiment (Supplementary Figure S3). This result is in agreement with observations of reduced cytotoxicity in previously reported docetaxel-fluorophore conjugates compared to free docetaxel[55] and is likely due to the increase of probe hydrophobicity that can alter cell permeability and microtubule affinity[49]. Taken together, the imaging results establish that the taxoid moiety behaves as expected to assemble itself onto microtubule structures in

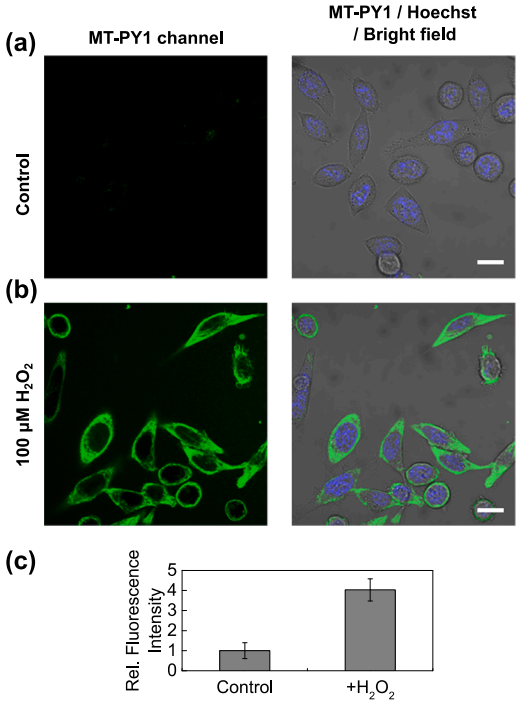


Figure 4. Fluorescence responses of MT-PY1 to exogenous addition of H_2O_2 in living cells. HeLa cells were incubated with 1 μM MT-PY1 for 0.5 h at 37 °C, followed by treatment with (a) vehicle control or (b) 100 μM H_2O_2 at 37 °C for 0.5 h; quantification is shown in (c). Scale-bar: 20 μm.

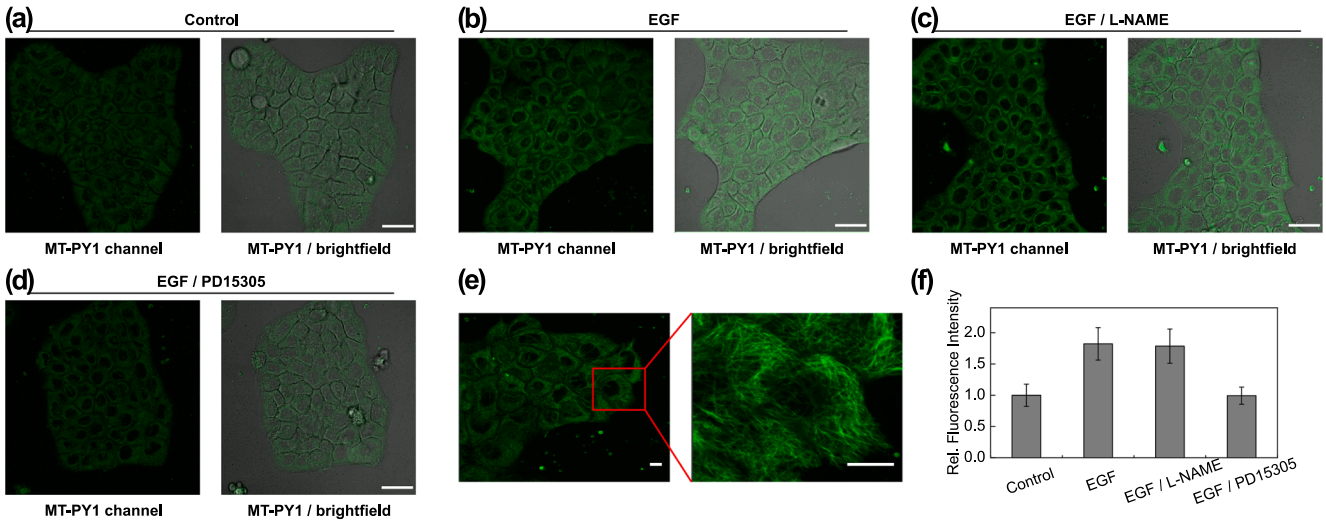


Figure 5. Fluorescence imaging of endogenous H_2O_2 generated by the EGF signaling pathway in live A431 cells. A431 cells incubated with 1 μM MT-PY1 for 0.5 h at 37 °C were treated with (a) vehicle control, (b) 100 ng/mL EGF, (c) 100 ng/mL EGF and 500 μM L-NAME or (d) 100 ng/mL EGF and 50 μM PD15305 for 30 min at 37 °C and imaged. (e) Zoomed-in images of A431 stained with MT-PY1 showing the high spatial resolution of this fluorescence probe. (f) Quantification of fluorescence intensity of a-d shown as mean ± s.d. Scale-bar: 50 μm.

living cells with negligible toxicity, which endows the MT-PY1 H_2O_2 probe with the ability to localize to microtubules.

We then evaluated the ability of MT-PY1 to respond to changes in H_2O_2 levels in living cells with exogenous incubation of ROS. Indeed, after loading live HeLa cells with MT-PY1, treatment with 100 μM H_2O_2 resulted in a significant fluorescence enhancement (Figure 4). More importantly, the probe retained its microtubule-localizing pattern after its turn-on response to H_2O_2, which establishes that the MT-PY1 reagent can sense changes in H_2O_2 localized to microtubules.

After establishing that MT-PY1 is suitable for monitoring changes in levels of H_2O_2 in cells with spatial resolution proximal to microtubules, we moved forward to testing the response of this activity-based sensing reagent to endogenous H_2O_2 produced by growth factor stimulation. We used A431 cells, a cancer cell line with high expression of the epidermal growth factor (EGF) receptor, as a biological model for endogenous peroxide production generated by EGF treatment. Upon treatment of MT-PY1-loaded A431 cells with EGF (100 ng/mL), we observed a significant fluorescence turn-on response (Figure 5a,b), which is in the same range with PY1 under similar conditions with higher amounts of EGF added (500 ng/mL). Moreover, when we treated the cells with both EGF and L-NAME, a nitric oxide synthase (NOS) inhibitor that can reduce generation of peroxynitrite and other RNS in cells, we observed a comparable fluorescence intensity signal compared to cells treated with EGF only (Figure 5c). This result indicates that peroxynitrite, which showed reactivity with MT-PY1 in *in vitro* assays, does not contribute to the observed turn-on response of the H_2O_2 probe in this biological context. In contrast, when we treated cells with EGF and PD15305, an EGF receptor inhibitor, we observed similar fluorescence to the untreated cells (Figure 5d), further confirming that the turn-on effect was indeed resulted from endogenous H_2O_2 generated in the EGF signaling pathway. In contrast to the punctate staining of PY1 in A431 cells, a higher-magnification image showed filament-localization pattern of the MT-PY1 probe, correlating with the microtubule-localizing property of this dye that give rise to even and spatially-resolved distribution in cytoplasm (Figure 5e).

To close, we have presented the design, synthesis, and properties of MT-PY1, a microtubule-targeting fluorescent probe for activity-based sensing of H_2O_2 in living cells. This reagent enables selective detection of changes in local H_2O_2 levels, including endogenous H_2O_2 produced by growth factor stimulation, with retention of microtubule targeting before and after ROS detection to preserve spatial information on redox signaling events. In addition to further applications of MT-PY1 and related reagents in deciphering the contributions of H_2O_2 to redox biology, this work provides a starting point for the design of a broader palette of activity-based sensing probes that utilize taxoid-targeting moieties to minimize probe diffusion before and after analyte detection.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.128252.

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