
Borinic acids as new fast-responsive triggers for hydrogen peroxide detection.

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Dedicated to Professor Jean-Marie Beau on the occasion of his 72nd birthday.

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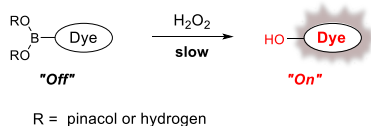
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Abstract: Detection of hydrogen peroxide (H₂O₂), which is responsible for numerous damages when overproduced, is crucial for a better understanding of H₂O₂-mediated signalling in physiological and pathological processes. For this purpose, various “off-on” small fluorescent probes relying on a boronate trigger have been developed. However, they suffer from low kinetics and do not allow for H₂O₂-detection with a short response time. Therefore, more reactive sensors are still awaited. To address this issue, we have successfully developed the first generation of borinic-based fluorescent probes containing a coumarin-scaffold. We determined the *in vitro* kinetic constants of the probe toward H₂O₂-promoted oxidation. We measured $1.9 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ as a second order rate constant, which is 10 000 faster than its boronic counterpart ($1.8 \text{ M}^{-1} \cdot \text{s}^{-1}$). This remarkable reactivity was also effective in a cellular context, rendering the borinic trigger an advantageous new tool for H₂O₂ detection.

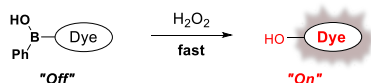
Reactive oxygen species (ROS) are involved in various physiological processes. In particular, hydrogen peroxide (H₂O₂) plays a critical role in the regulation of numerous biological activities as a signalling molecule.^[1] However, aberrant production or accumulation of H₂O₂ leads to oxidative stress conditions, which can cause lesions associated with aging, cancer^[2] and several neurodegenerative diseases such as Alzheimer's or Parkinson's.^[3] Differentiation of physiological or abnormal conditions is closely connected with slight changes in H₂O₂ levels. However, the generation and degradation of H₂O₂ are variable within different cellular compartment, and this small molecule is highly diffusive, rendering difficult the detection of small H₂O₂ fluctuations and the study of its spatial and temporal dynamics. Therefore, the development of selective and sensitive tools allowing H₂O₂ detection in a biological context represents a great challenge for a better understanding of H₂O₂-mediated signalling in physiological and pathological processes.

Among numerous strategies developed to detect H₂O₂, “off-on” small fluorescent probes have attracted particular attention due to their easy implementation, high expected signal-to-noise ratio, and compatibility with standard equipment present in cellular biology research environments.^[4] The fluorescence of such probes is triggered or modified by H₂O₂-mediated transformation of a suitable chemical moiety. Several approaches have been explored including probes based on arylsulfonyl esters hydrolysis,^[5] oxidation of arylboronates,^[6] Baeyer-Villiger oxidation of diketones,^[7] Tamao oxidation of silanes^[8] or a tandem Payne-Dakin reaction.^[9] Among them, probes based on the boronate esters oxidation pioneered by Chang are the most explored sensors, due to their remarkable stability, low toxicity profile, ease of preparation and their specificity towards H₂O₂.^[10] Upon reaction with H₂O₂, these compounds undergo an oxidative conversion into aryl borate esters that further hydrolyse into the corresponding fluorescent phenols along with borate esters or boric acid (Scheme 1A). This conversion turns on probe fluorescence either directly or *via* the degradation of a self-immolative spacer. This chemospecific and biologically compatible reaction allowed for developing highly selective fluorescent probes for H₂O₂ imaging in cells.^[11] However, H₂O₂-triggered conversion of boronic acids to fluorescent phenols is still not satisfactory since most of these probes have second order reaction rate constants of $0.1\text{-}1.0 \text{ M}^{-1} \cdot \text{s}^{-1}$.^[12] In cells, H₂O₂ is present in the 1-100 nM concentration range in physiological conditions and could reach up to 100 μM under oxidative stress conditions.^[13] Therefore, most of the boronate-based probes need an incubation time longer than 30 minutes to detect a H₂O₂ concentration of 100 μM. Thus, alternative H₂O₂ sensors with rapid reaction rates allowing real-time monitoring of H₂O₂ are still expected.

A. Current boronate-based H₂O₂ sensors



B. This study: borinic-based H₂O₂ sensor



Scheme 1. Principle of “Off-On” fluorescent probes for H₂O₂ detection. **A.** Current boronate-based H₂O₂ sensors. **B.** This study: a new borinic-based H₂O₂ sensor.

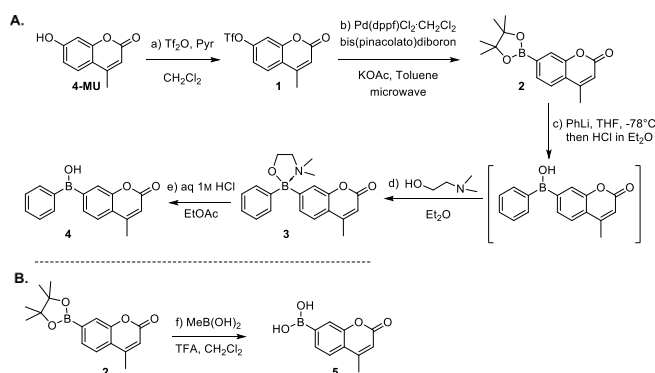
To address this issue, we envisioned the use of borinic acids, which due to electronic effects could be more prone to rapid oxidation compared to their boronic acid counterpart. These structures have been mainly exploited as catalysts for their electrophilic properties in various reactions such as epoxide ring opening,^[14] hydrosilylation,^[15] transamidation,^[16] aldol reaction,^[17] selective monoalkylation of diol^[18] or regioselective glycosylation reactions.^[19] Surprisingly, the reactivity of these borinic species, remains underexplored, probably due to their limited synthetic routes.^[20] They were usually obtained through the addition of strong organometallic reagents (RLi/RMgBr) onto boron-based electrophiles such as trialkylborates, boron halides, diborane or boronate esters. To date, a detailed study of the reactivity of borinic acids towards oxidation including reaction with hydrogen peroxide has not been reported and their use as a trigger for the direct release of a probe has never been considered.

Herein, we report for the first time the design and the synthesis of a new borinic sensor for the direct detection of the hydrogen peroxide molecule (Scheme 1B). We establish a detailed kinetic analysis of the H₂O₂-promoted oxidation of this new fluorogenic borinic acid as well as a comparative study with its corresponding boronic analogue, which has never been carried out yet. Furthermore, we demonstrate the efficiency of the borinic trigger toward H₂O₂-mediated oxidation in a cellular context.

First, in order to validate the general approach, we compared the reactivity of simple model compounds towards H₂O₂, i.e., phenylboronic acid synthesized from addition of phenyl lithium onto pinacol phenylboronate^{[20b][21]} and the commercially available phenylboronic acid. These two compounds were submitted to oxidation with one equivalent of H₂O₂ in deuterated PBS buffer (pH 7.4). The reaction progress was monitored using ¹H NMR spectroscopy. These early experiments were extremely encouraging since we observed a complete oxidation of the phenylboronic acid into phenol and phenylboronic acid within 2 minutes whereas 2 hours were required for full conversion of the phenylboronic acid into phenol under the same conditions (Scheme S1).

Capitalizing on these preliminary results, we focused on the synthesis of a borinic acid-based fluorogenic probe containing a phenyl moiety and a simple coumarin scaffold in order to validate our proof of concept. The synthetic route involved a

four-step sequence in which the key reaction relies on the addition of phenyl lithium onto a coumarin-based pinacol boronic ester (Scheme 2A). Thus, treatment of 4-methylumbelliferone (**4-MU**) with trifluoromethanesulfonic anhydride afforded the corresponding triflate **1** in 97% yield, which was subjected to a Pd-catalyzed cross coupling reaction with bis(pinacolato)diboron under microwave irradiation according to Chang's conditions.^{[11b][22]} The resulting pinacol boronic ester **2** was then reacted with a solution of phenyl lithium to afford the borinic acid,^[23] which was immediately converted into the corresponding *N,N*-dimethylethanolamine ester **3**.^[24] This compound was isolated by precipitation in 24% yield over two steps.^[25] Acidic hydrolysis provided the expected boronic acid **4** in 67% yield. The corresponding 4-methyl coumarin boronic acid **5** was also synthesized for comparison. It was easily obtained from the pinacol ester using recently reported transesterification reaction with methylboronic acid (Scheme 2B).^[26]



Scheme 2. Synthesis of 4-methylcoumarin-based boronic acid (A). Conditions: a) Tf₂O (1.1 equiv), Pyr (2.2 equiv), CH₂Cl₂, RT, 2 h, 97%; b) bis(pinacolato)diboron (1.2 equiv), KOAc (1.5 equiv), Pd(dppf)Cl₂·CH₂Cl₂ (10 mol%), toluene, microwave irradiation, 120°C, 3 h, 65%; c) PhLi in *n*-Bu₂O (c 1.9M, 1.1 equiv), -78°C, 4 h then HCl in Et₂O (c 2M, 7 equiv), -78°C to RT; d) *N,N*-dimethylethanolamine (2 equiv), Et₂O, RT, 12 h, 24%; e) HCl, EtOAc, RT, 30 min, 67%; **Synthesis of 4-methylcoumarin boronic acid (B):** f) MeB(OH)₂, TFA, CH₂Cl₂, RT, 4 h, 87%.

Hydrogen peroxide-mediated oxidation of **4** and **5** was first monitored by ¹H NMR spectroscopy in deuterated PBS buffer (pH 7.4). As previously observed with the phenyl-based model compounds, the borinic derivative was found to be more reactive than its boronic counterpart. Thus, the addition of H₂O₂ triggers full oxidation of the borinic acid **4** in less than 2 minutes. In contrast, the boronic acid **5** is gradually converted into **4-MU** to reach complete conversion within 2.5 hours (Scheme S2). Due to the dissymmetrical nature of the boronic acid probe, the oxidative rearrangement of **4** leads to the formation of two different alcohol/boronic acid couples, i.e. either phenol/4-methylcoumarin boronic acid **5** as the undesired cleavage products, or **4-MU**/phenylboronic acid as the expected cleavage products. The ratio of both was determined by ¹H NMR integration and was evaluated to be 80/20 against the direct release of **4-MU** (Figure 1).

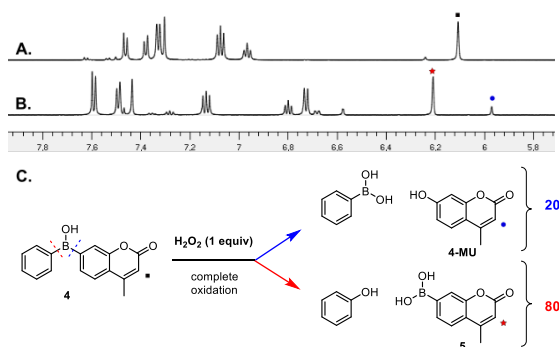


Figure 1. Regioselectivity of the oxidation of probe **4** determined by ^1H NMR spectroscopy (600 MHz) in deuterated PBS buffer (pH 7.4, 0.2% DMSO). ^1H NMR spectra of the boronic acid **4** (A) and **4** with H_2O_2 -urea complex (1 equiv) after 2 minutes (B). (C) The two different couples of alcohol/boronic acid resulting from the oxidation reaction. 4-MU= 4-methylumbelliferone.

Having demonstrated the higher reactivity of the borinic-based probe, we then undertook the accurate determination of the kinetic constants of the oxidation reactions (Figure 2A). To calculate these parameters, we used real-time measurement of the fluorescence emission performed under pseudo-first order conditions (excess of oxidant). Initial H_2O_2 -promoted oxidation experiments were conducted with boronic acid **5** at different temperatures and H_2O_2 concentrations. As expected, reaction with H_2O_2 leads to an increase in fluorescence emission at 450 nm indicating the release of the **4-MU** chromophore (Figure 2C).

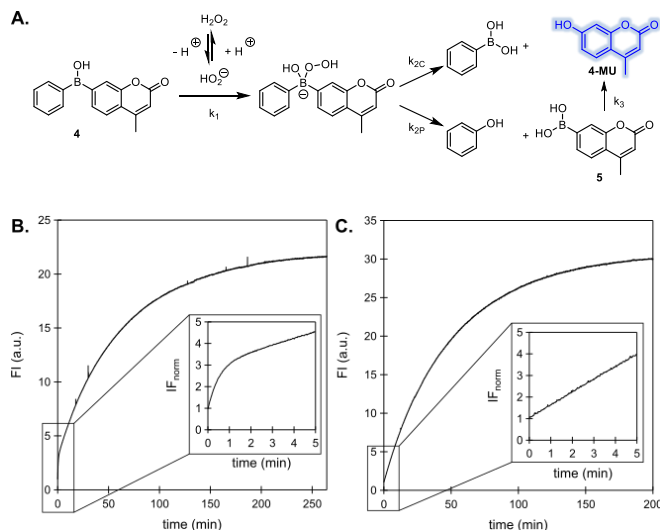


Figure 2. Kinetic study of oxidation of probe **4** and boronic acid **5**. (A) Simplified kinetic scheme of oxidation of the boronic acid **4**. (B,C) Time-dependent fluorescence intensity evolution of 5 μM **5** (C) or **4** (B). Upon addition of 100 μM H_2O_2 -urea solution in PBS buffer 1X (pH 7.4, 0.04% DMSO) at 310 K (pseudo-first order conditions). The fluorescence emission was normalized and was recorded at $\lambda_{\text{em}} = 450$ nm ($\lambda_{\text{ex}} = 405$ nm).

For kinetic analysis of the experimental data, a model including the different steps involved in the **4-MU** release was taken into account allowing to determine the second order rate constant k_3 associated with the oxidation as $1.8 \text{ M}^{-1}\cdot\text{s}^{-1}$ and $6.3 \text{ M}^{-1}\cdot\text{s}^{-1}$ at 293 and 310 K, respectively (Figure S4). These results are consistent with those previously reported in the literature.^[27]

In comparison, oxidation of borinic derivative **4** shows a different behaviour. Upon addition of H_2O_2 , fluorescence rapidly increases, which suggests an oxidation of the borinic acid probe **4** at the minute timescale affording directly **4-MU** through the desired but minor oxidation pathway. After a few minutes, fluorescence increases more slowly, at a time scale which is in line with the formation of **4-MU** from the boronic acid **5** obtained as a major product from oxidizing probe **4** (Figure 2B). Upon considering that several reactions are rapid at the timescale of the overall oxidation process (ESI[†]), we could account for the oxidation of the borinic acid **4** with the reduced mechanism shown in Figure 2A. After integration of the kinetic results obtained above for the boronic acid **5**, we extracted 1.9×10^4 and $4.2 \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$ for the rate constant k_1 at 293 and 310 K, respectively (see ESI[†] for the determination). These results point to an oxidation of the borinic trigger which is about 10 000 times faster than for its boronic counterpart.

Based on these promising results, we furthermore investigated the ability of the borinic-based probe to detect hydrogen peroxide in a cellular environment. For this purpose, we used COS7 cells stably expressing the two membrane subunits of the NADPH oxidase (COS7^{gp91-p22} cells). Upon transfection with a chimeric protein named trimera and composed of the domains necessary for the activation of the oxidase, the cells continuously produce superoxide radical anion (see SI for details on the trimera).^[28] Since superoxide spontaneously dismutates into H_2O_2 in aqueous buffer,^[29] these cells constitute an interesting model to investigate the sensitivity of the probe for endogenous ROS detection. A preliminary experiment was performed with non-transfected COS7^{gp91-p22} cells in the presence of 10 μM borinic acid **4**. Upon addition of an exogenous solution of hydrogen peroxide (final concentration 100 μM), fluorescence intensity at 450 nm increases rapidly within a few minutes and then more slowly, as observed during the *in vitro* kinetic study (Figure S8). Control experiment performed without H_2O_2 resulted in an absence of fluorescence signal providing evidence for the reactivity of the borinic acid toward exogenous hydrogen peroxide in a cellular context. We then focused on the ability of probe **4** to detect endogenous H_2O_2 generated by NADPH oxidase. Thus, fluorescence signal was monitored upon addition of the borinic probe **4** and its boronic counterpart **5** on either transfected cells, which continuously produce hydrogen peroxide, or non-transfected cells as control experiments (Figure 3).

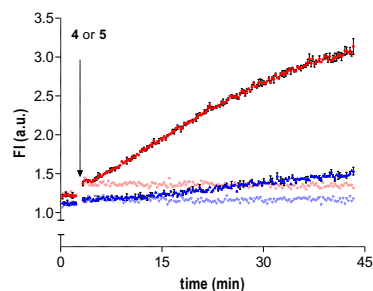


Figure 3. Fluorophore release in a cellular context. Time-dependent fluorescence intensity of 10 μM borinic probe **4** (red curve) or 10 μM boronic acid **5** (blue curve) in transfected COS7^{gp91/p22} cells. Negative controls in non-transfected COS7^{gp91/p22} cells for the borinic probe **4** (light red curve) and the boronic acid **5** (light blue curve). Data were recorded in PBS buffer 1X (pH 7.4, 0.02% DMSO) at 310 K, at $\lambda_{\text{em}} = 450$ nm ($\lambda_{\text{ex}} = 360$ nm).

In the case of cells expressing an activated oxidase, the borinic acid probe **4** generated a significantly more intense fluorescence signal than the boronic probe **5** under the same conditions. The ratio of the two slopes indicates that **4-MU** is released 10-fold faster with the borinic probe than with the boronic one, which is in a reasonable agreement with the theoretical value determined by our kinetic studies (see ESI†). Moreover, no fluorescence increase was observed either using non-transfected COS-7^{gp91/p22} cells (Figure 3, light curves), or in the presence of diphenyliodonium chloride (DPI), a NADPH oxidase inhibitor, or catalase, an enzyme which catalyses H₂O₂ dismutation (Figure S9). All these control experiments provide evidences for the reactivity of the probe **4** toward endogenous hydrogen peroxide. These results clearly support the borinic trigger as a suitable tool for endogenous H₂O₂ detection in a physiological environment.

In conclusion, we have described the first example of a borinic-based probe enabling direct fluorophore release upon H₂O₂-mediated oxidation. Through detailed kinetic studies, we demonstrated that this coumarin-containing borinic sensor responded to H₂O₂ in an unprecedented short time, even when H₂O₂ is endogenously produced by cells. Optimizations are now underway in the laboratory to control the regioselectivity of probe oxidation and get improved sensors in terms of spectral properties and brightness for imaging. Noteworthy this work has revealed the superior reactivity of the borinic trigger toward H₂O₂-promoted oxidation compared to the corresponding boronic counterpart, rendering the borinic trigger a valuable tool for hydrogen peroxide detection. Such favorable features should also secure promising applications of this new trigger for drug release and theranostics.

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Keywords: borinic acid • fluorescent probes • H₂O₂ detection • oxidative stress • kinetics

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