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## Identification of peroxyxynitrite by profiling oxidation and nitration products from mitochondria-targeted arylboronic acid

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### Summary

The development of boronic probes enabled reliable detection and quantitative analysis of hydrogen peroxide, other nucleophilic hydroperoxides, hypochlorite and peroxyxynitrite. The major product, in which boronate moiety of the probe is replaced by the hydroxyl group, is however common for all those oxidants. Here, we describe how *ortho*-isomer of mitochondria-targeted phenylboronic acid can be used to detect and differentiate peroxyxynitrite-dependent and independent probe oxidation. This method highlights detection and quantification of both the major, phenolic product and the minor, peroxyxynitrite-specific cyclic and nitrated products of probe oxidation.

### Keywords

Hydrogen peroxide; Peroxyxynitrite; Mitochondria-targeted probes; Boronic probes; *o*-MitoPhB(OH)<sub>2</sub>; HPLC-MS

## 1. Introduction

Boronate-based probes were developed over the last decade for detection of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and peroxyxynitrite (ONOO<sup>-</sup>) in biological systems (1–3). Mitochondria-targeted boronate probes were developed to monitor hydrogen peroxide in mitochondria (4–8). Boronates react directly and stoichiometrically with both H<sub>2</sub>O<sub>2</sub> and ONOO<sup>-</sup> (9,10). This is in contrast to more classical fluorogenic probes (e.g. dichlorodihydrofluorescein, DCFH and dihydrorhodamine, DHR), which need the catalyst (e.g. iron, heme proteins) or react with the products of ONOO<sup>-</sup> decomposition (11–13). Additionally, in contrast

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to boronates, both DCFH and DHR form intermediate radical species that reduce oxygen to superoxide radical anion (11,14). However, most boronates lack specificity needed to distinguish between hydrogen peroxide, other nucleophilic hydroperoxides and peroxyxynitrite in a complex biological system, as discussed below (2,9,15–17). In a simple system when bolus amounts of the reactants are quickly mixed this limitation can be overcome due to significant differences of the reaction rate constants. The rate constant of the reaction of boronates with peroxyxynitrite is six orders of magnitude higher than of the analogous reaction with hydrogen peroxide (2,9). For example, under the conditions when the reaction with  $\text{ONOO}^-$  is completed within 100 ms, the reaction with  $\text{H}_2\text{O}_2$  was not completed even after 12 h (9). The situation is, however, different when the oxidants react with the probe in biological systems, with the oxidants continuously produced during the incubation with the probe. Therefore, we proposed the use of specific inhibitors of oxidants formation and/or specific scavengers of the oxidants to differentiate between different species responsible for oxidation of boronate probes in cells (2,18). We have previously reported a formation of both major, phenolic product (*o*-MitoPhOH) and minor, nitrated product (*o*-MitoPhNO<sub>2</sub>) during the reaction of peroxyxynitrite with *ortho*-isomer of mitochondria-targeted phenylboronic acid (*o*-MitoPhB(OH)<sub>2</sub>, Fig. 1) (19,20). More recently, we have identified an additional, more abundant minor product, *cyclo-o*-MitoPh (Fig. 1) (21,22). These minor products are specific for  $\text{ONOO}^-$  reaction and are not produced by any other oxidant. Therefore the proposed method provides also an opportunity to distinguish between two biological nitrating pathways: peroxyxynitrite-dependent and myeloperoxidase/hydrogen peroxide/nitrite-dependent (21). Here, we show how this unique chemistry can be utilized to selectively detect peroxyxynitrite in cellular systems (23,24). The method is based on probing the oxidants ( $\text{ONOO}^-$  and  $\text{H}_2\text{O}_2$ ) in cells by incubation of cells with *o*-MitoPhB(OH)<sub>2</sub> followed by extraction and HPLC-MS/MS analysis of the products formed (21,25). We describe the protocol for probe preparation, processing of biological samples and HPLC-MS-based analysis of the products formed.

## 2. Materials

### 2.1. Components for the synthesis of *o*-MitoPhB(OH)<sub>2</sub>

1. 2 g of triphenylphosphine.
2. 200 ml of anhydrous diethyl ether.
3. 1 g of 2-(bromomethyl)phenylboronic acid.
4. 100 ml of dichloromethane
5. Argon gas.

### 2.2. Cell incubation components

1. Cell growth medium (for RAW 264.7 cells): DMEM containing 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin).
2. Assay medium: DPBS supplemented with 5.5 mM glucose and 0.33 mM pyruvate (*see* Note 1).

3. Solution of 50 mM *o*-MitoPhB(OH)<sub>2</sub> (for description of synthesis see 3.1.) in DMSO.

### 2.3. Cell extraction components

1. Cell lysis buffer: 10 ml DPBS containing 0.1% Triton X100 spiked with 1 μM mixture of internal standards, place on ice (can be stored at 4 °C) (*see* Note 2).
2. 100 ml of ice-cold acetonitrile containing 0.1% (v/v) formic acid.
3. 10 ml of ice-cold acetonitrile containing 0.1% (v/v) formic acid and 1 μM mixture of internal standards, place on ice (can be stored for a short term at 4 °C).
4. Protein assay reagent (Bradford reagent).
5. BSA in lysis buffer: 20 mg/ml BSA. Prepare a series of BSA solutions by serial dilutions with the final concentrations of 0.5, 1.0, 1.5, 2, 3, 4, 5, 7 and 10 mg/ml. Keep the solutions on ice.

### 2.4. HPLC analysis components

1. HPLC mobile phase: 0.1 % formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) (*see* Note 3).
2. Solution of *o*-MitoPhNO<sub>2</sub> (50 mM) in DMSO (*see* Note 4).
3. Solution of *o*-MitoPhB(OH)<sub>2</sub> (50 mM) in DMSO.
4. Solution of *o*-MitoPhOH (50 mM) in DMSO (*see* Note 4).
5. Solution of *cyclo-o*-MitoPh (50 mM) in DMSO (*see* Note 5).
6. Water:acetonitrile (3:1) mixture containing 0.1% (v/v) formic acid, spiked with 1 μM mixture of the internal standards. This will be a solvent for preparation of standards for HPLC calibration.
7. Mixture of the standards of *o*-MitoPhB(OH)<sub>2</sub>, *o*-MitoPhOH and *o*-MitoPhNO<sub>2</sub> and *cyclo-o*-MitoPh (0.1 mM each) in water:acetonitrile (3:1) solvent containing 0.1 % formic acid spiked with 1 μM mixture of the internal standards. Prepare serial dilutions down to 1 nM concentration. Use the solvent for standards prepared in previous step.

## 3. Methods

### 3.1. Preparation of *o*-MitoPhB(OH)<sub>2</sub>

1. The preparation of the *o*-MitoPhB(OH)<sub>2</sub> probe should be carried out inside a hood with well-working ventilation!
2. Prepare a solution of triphenylphosphine (1.048 g, 4.0 mmol) in anhydrous diethyl ether (30 ml).
3. Add 2-(bromomethyl)phenylboronic acid (0.856 g, 4.0 mmol) with constant stirring under argon atmosphere.

4. Stir the reaction mixture for 46 h (using magnetic stirrer) at room temperature under argon atmosphere.
5. A crystalline precipitate of bromide salt of *o*-MitoPhB(OH)<sub>2</sub> should be obtained.
6. Filter the suspension. Keep the solid and discard the filtrate.
7. Wash the solid with anhydrous diethyl ether (30 ml). Filter the suspension again and keep the solid.
8. Dissolve the solid in dichloromethane (in a minimum volume, < 5 ml) and precipitate the compound back by addition of diethyl ether (1:10). Filter the powder and keep the solid. Repeat this step once more.
9. Leave the solid to dry. A white powder should be obtained with the melting point of 225–226 °C. Test the identity and purity of the synthesized probe (see Note 6).

### 3.2. Cell incubation with the probe

1. Prepare the cells according to the experimental conditions to be tested for peroxynitrite formation (*see* Note 1).
2. Add *o*-MitoPhB(OH)<sub>2</sub> to obtain the final concentration of 50 μM (*see* Notes 7 and 8).
3. Incubate the cells for 1 h.
4. Collect an aliquot of the medium (0.1 ml) in 1.5 ml microcentrifuge tube and freeze in liquid nitrogen.
5. Remove the rest of medium and wash the cells twice with ice-cold DPBS.
6. Add 1 ml of ice-cold DPBS and harvest the cells, place the cell suspension in 1.5 ml microcentrifuge tube and spin down the cells by quick centrifugation. Remove the supernatant and freeze the cell pellet in liquid nitrogen.
7. Frozen cell pellets and media can be stored at –80 °C for at least one week before analysis.

### 3.3. Extraction of the products

#### 3.3.1. Cell pellets

1. Preload one set of 1.5 ml microcentrifuge tubes with 0.1 ml of 0.1% formic acid in acetonitrile and place on ice.
2. Preload a second set of 1.5 ml microcentrifuge tubes with 0.1 ml of 0.1% formic acid in water and place on ice.
3. Prepare a clear-bottom 96-well plate for protein assay and place on ice.
4. Place the tubes with frozen cell pellets on ice.
5. Add 0.2 ml of the lysis buffer spiked with a mix of internal standards (1 μM each) and lyse the cells by 10 syringe strokes using a 0.5 ml insulin syringe with the needle 28 Gauge × 0.5 inch (0.36 mm × 13 mm).

6. Transfer 0.1 ml of the cell lysates into the tubes containing ice-cold 0.1% formic acid in acetonitrile, vortex for 10 s and place on ice. Transfer  $3 \times 2 \mu\text{l}$  of the cell lysate aliquots into 3 wells on 96-well plate for the protein assay.
7. Incubate the mixtures of cell lysates with acetonitrile for 30 min on ice.
8. During incubation measure the protein concentration in the cell lysates using Bradford assay and plate reader with absorption detection (*see* Note 9).
9. Vortex the tubes again for 5 s and centrifuge for 30 min at 20,000*g* at 4 °C.
10. Place the tubes back on ice and transfer 0.1 ml aliquots of the supernatants into the second set of tubes, containing 0.1% formic acid in water.
11. Vortex the tubes for 5 s and centrifuge for 15 min at 20,000*g* at 4 °C.
12. Transfer 0.15 ml of the supernatants into HPLC vials preloaded with conical inserts, seal the vials and place on ice. Once all solutions have been transferred, place the vials in HPLC autosampler pre-cooled to 4 °C.

### 3.3.2. Media

1. Preload one set of 1.5 ml microcentrifuge tubes with 0.1 ml of 0.1% formic acid in water and place on ice.
2. Place the tubes with frozen media on ice.
3. Add 0.1 ml of the ice-cold 0.1% formic acid in acetonitrile spiked with internal standards (1  $\mu\text{M}$  each) to each tube, vortex for 10 s and place on ice.
4. Incubate the mixtures of media with acetonitrile for 30 min on ice.
5. Vortex the tubes again for 5 s and centrifuge for 30 min at 20,000*g* at 4 °C.
6. Place the tubes back on ice and transfer 0.1 ml aliquots of the supernatants into the tubes containing 0.1% formic acid in water.
7. Vortex the tubes for 5 s and centrifuge for 15 min at 20,000*g* at 4 °C.
8. Transfer 0.15 ml of the supernatants into HPLC vials preloaded with conical inserts, seal the vials and place on ice. Once all solutions have been transferred, place the vials in HPLC autosampler pre-cooled to 4 °C.

### 3.4. HPLC-MS/MS analysis of the extracts

1. Install the column Kinetex Phenyl-Hexyl 50 mm  $\times$  2.1 mm, 1.7  $\mu\text{m}$  (Phenomenex) in the HPLC-MS/MS system. The column should be equipped with a UHPLC column filter or guard column to extend the column lifetime.
2. Equilibrate the column with the mobile phase (75% of mobile phase A and 25% mobile phase B).
3. Setup the HPLC-MS/MS method and detection parameters according to Tables 1 and 2, respectively (*see* Note 10).

4. Test the system by three injections of standards (10  $\mu\text{M}$ ) for the reproducibility of retention times and peak intensities for all analytes and internal standards, as shown in Fig. 2.
5. Run the analysis of the batch of samples.
6. Include the system and column wash with water:methanol (1:1) mixture at the end of batch.
7. Quantify each analyte based on the specific MRM transitions and calibration curves constructed in the concentration range relevant to the samples analyzed (*see* Note 11).
8. When appropriate, normalize the concentrations of analytes to the protein levels in cell lysates, as determined by Bradford method.
9. Increase in peak intensities of both *o*-MitoPhOH, *cyclo-o*-MitoPh, and *o*-MitoPhNO<sub>2</sub> (Fig. 3) indicates formation of ONOO<sup>-</sup>, while formation of *o*-MitoPhOH, but not *o*-MitoPhNO<sub>2</sub> (Fig. 4) indicates the presence of other oxidants, most commonly H<sub>2</sub>O<sub>2</sub> (*see* Note 12).

#### 4. Notes

1. The medium used may be selected according to experimental design, and cell culture needs. However, it is preferred that when monitoring extracellular ONOO<sup>-</sup> and/or H<sub>2</sub>O<sub>2</sub>, the components of the medium capable of scavenging those oxidants should be avoided, if possible. For example, pyruvate in the medium may efficiently compete with boronate probes for H<sub>2</sub>O<sub>2</sub>.
  - a. Internal standards for *o*-MitoPhB(OH)<sub>2</sub>, *o*-MitoPhOH, and *o*-MitoPhNO<sub>2</sub> are their isotopologs containing deuterated triphenylphosphonium moiety (Fig. 2). These are not commercially available but can be synthesized in analogous ways as *o*-MitoPhB(OH)<sub>2</sub>, *o*-MitoPhOH, and *o*-MitoPhNO<sub>2</sub>, (*see* Note 4, for description of synthesis of *o*-MitoPhB(OH)<sub>2</sub> *see* 3.1.), but starting with commercially-available triphenylphosphine-*d*<sub>15</sub>. As *cyclo-o*-MitoPh is not synthesized from triphenylphosphine, we do not have deuterated isotopolog for use as an internal standard. Instead, we use (2-methylbenzyl)triphenylphosphonium (*o*-MitoPhCH<sub>3</sub>) as an internal standard for *cyclo-o*-MitoPh (Fig. 2).
2. LC-MS (preferably UHPLC-MS) grade solvents and formic acid should be used. After preparation, mobile phase should be passed through 0.2  $\mu\text{m}$  filter. Prepare only the amount of mobile phase, which is necessary for the experiment. Do not store the mobile phase for longer than 2–3 days to avoid any growth of the biological matter.
3. The standards of the products, *o*-MitoPhOH and *o*-MitoPhNO<sub>2</sub> are commercially available, but can be also synthesized. The phenolic product can be prepared by reaction *o*-MitoPhB(OH)<sub>2</sub> with hydrogen peroxide, followed by addition

of catalase to remove excess  $\text{H}_2\text{O}_2$ . The nitrated product can be synthesized in an analogous protocol as described for *o*-MitoPhB(OH)<sub>2</sub>, but starting with 2-nitrobenzyl bromide instead of 2-(bromomethyl)phenylboronic acid.

4. *Cyclo-o*-MitoPh is not commercially available should be synthesized according to the published method (21). If only small amounts of *cyclo-o*-MitoPh are needed, it may be also produced by reacting *o*-MitoPhB(OH)<sub>2</sub> with ONOO<sup>-</sup>, followed by HPLC-based purification. The identity of the isolated fraction and the extent of contamination by *o*-MitoPhNO<sub>2</sub> need to be determined. In our experience, sufficient chromatographic resolution between the peaks of *cyclo-o*-MitoPh and *o*-MitoPhNO<sub>2</sub> can be obtained using Raptor Biphenyl column (Restek, Bellefonte, PA, USA; 100 mm × 2.1 mm, 2.7 μm).
5. Purity of synthesized *o*-MitoPhB(OH)<sub>2</sub> should be tested by HPLC and the compound repurified, if needed. The identity should be confirmed by NMR (20,26) and HRMS (C<sub>25</sub>H<sub>23</sub>BO<sub>2</sub>P<sup>+</sup>, m/z = 397.1535) (19,20) analyses.
6. The concentration of *o*-MitoPhB(OH)<sub>2</sub> used for probing of ONOO<sup>-</sup> should be chosen so as it does not interfere with mitochondrial function. We did not observe significant effects of 50 μM *o*-MitoPhB(OH)<sub>2</sub> on the rate of oxygen consumption by RAW 264.7 cells or MiaPaCa-2 pancreatic cancer cells (25).
7. When exposing cells to *o*-MitoPhB(OH)<sub>2</sub> it is preferred to add the medium containing the probe, rather than directly adding a solution of concentrated *o*-MitoPhB(OH)<sub>2</sub> in DMSO, to avoid local exposure of cells to high concentrations of DMSO.
8. If plate reader with absorption detection is not available, protein measurements can be carried out using regular spectrophotometer. The volume of cell lysate needed for the assay may be higher, depending on the volume of the spectrophotometer cell.
9. To protect the detector, only portion of eluate is flowed into mass detector. This is achieved by using a diverter valve, which directs the flow into waste before 1 min and after 4 min after injection. Between 1 min and 4 min the flow is directed into the detector and the signals recorded.
10. For each compound two MRM transitions (pairs of parent ion/daughter ion) are recorded. The primary (dominant) transition is used for quantification, while the secondary (reference) transition is used for confirmation of the identity of the analyte. If the ratio of reference to dominant MRM transitions (Table 2) is outside the range allowed (typically ± 30%), the peak is rejected and not used for quantification. Due to similar m/z values of *o*-MitoPhB(OH)<sub>2</sub> and *o*-MitoPhNO<sub>2</sub>, there is a small peak of the boronate appearing in the channel of the nitro derivative (Fig. 2, a peak at 1.55 min), which may show up as the dominant peak, when *o*-MitoPhB(OH)<sub>2</sub> is in high excess comparing to *o*-MitoPhNO<sub>2</sub> (Fig. 3). This peak is not however observed in the reference channel, exemplifying the usefulness of the reference transitions for peak identification.



11. The identity of the oxidizing species can be tested by application of specific inhibitors and/or scavengers and/or identification of ONOO<sup>-</sup>-specific products (13;15). Confirmation of peroxynitrite involvement may be obtained by testing the inhibitory effects of inhibitors of nitric oxide synthase, for example L-NAME (18,20). Detection of ONOO<sup>-</sup> marker products, *cyclo-o*-MitoPh and *o*-MitoPhNO<sub>2</sub>, is sufficient to confirm the involvement of ONOO<sup>-</sup> in probe oxidation (21), as shown in Fig. 3. The inhibitory activity of catalase on the yield of *o*-MitoPhOH and the lack of formation of *o*-MitoPhNO<sub>2</sub> (Fig. 4) indicates the involvement of H<sub>2</sub>O<sub>2</sub> and not ONOO<sup>-</sup> in probe oxidation.

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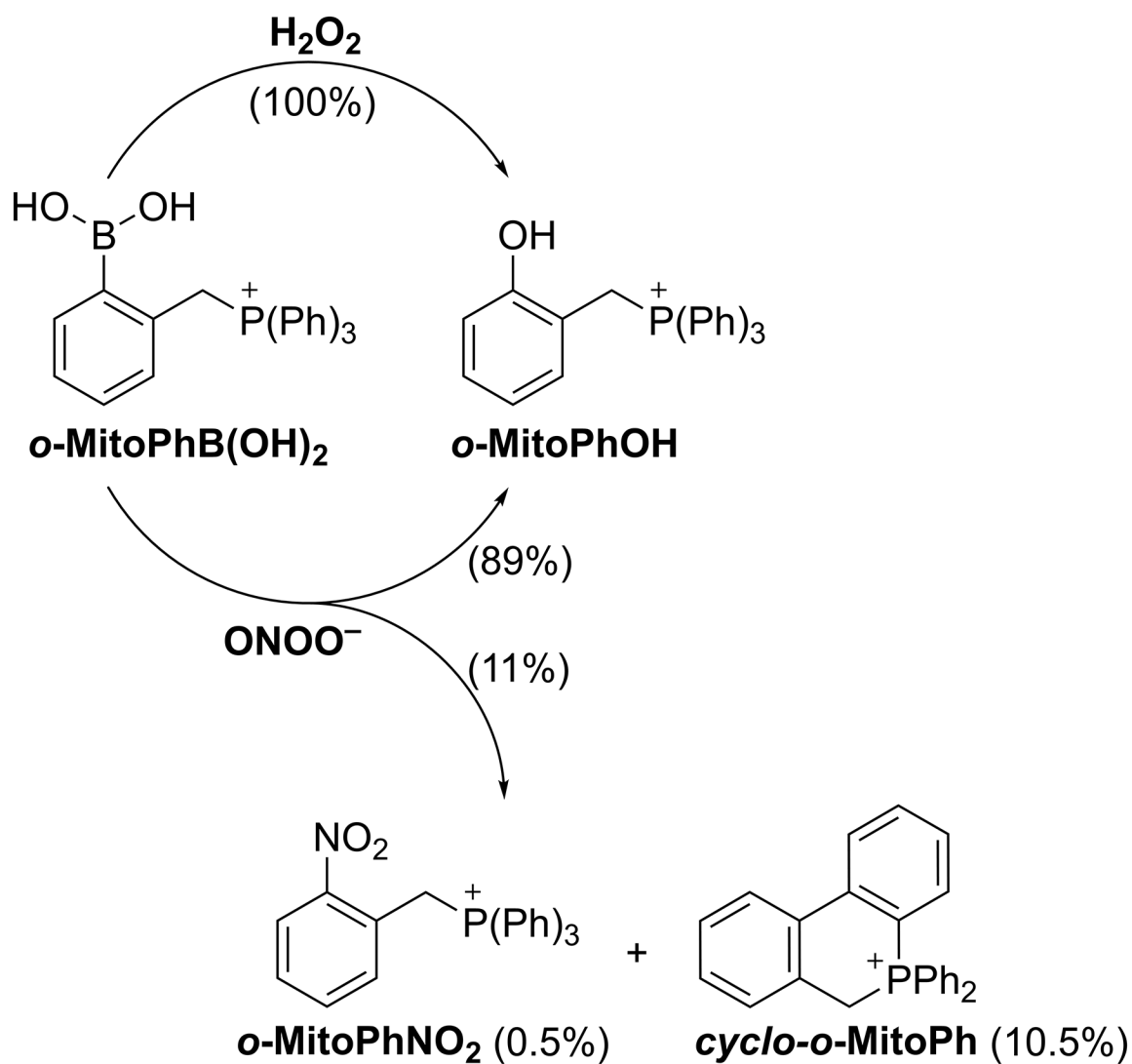
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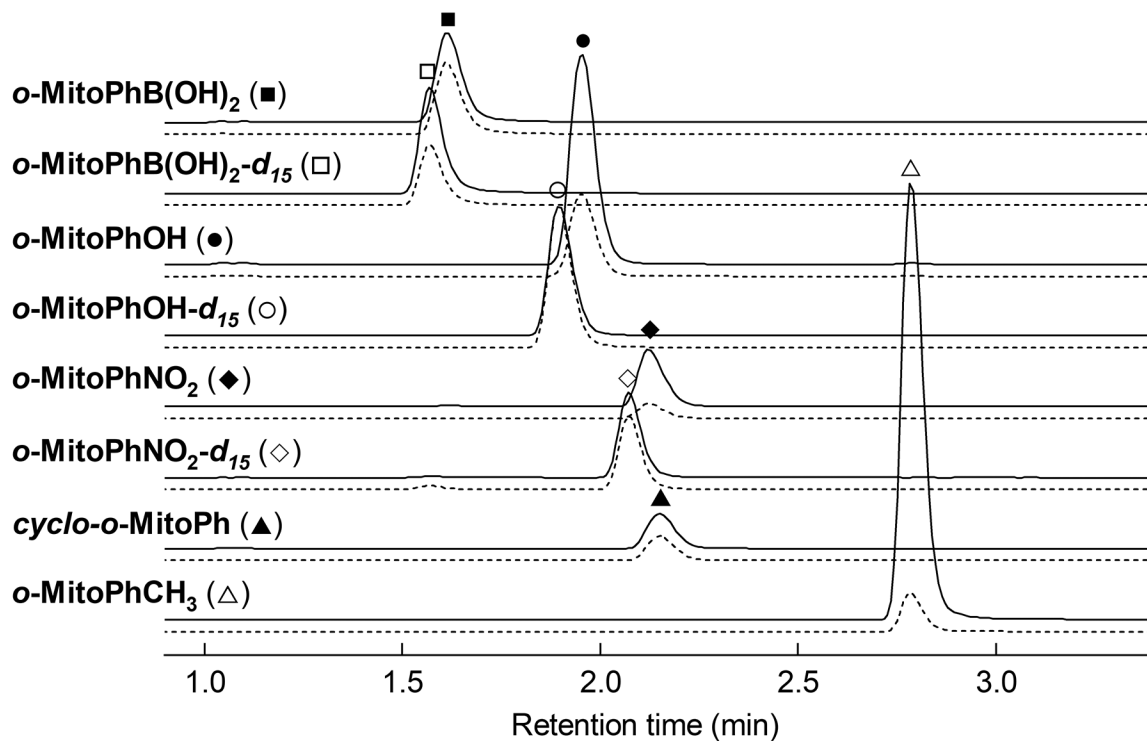


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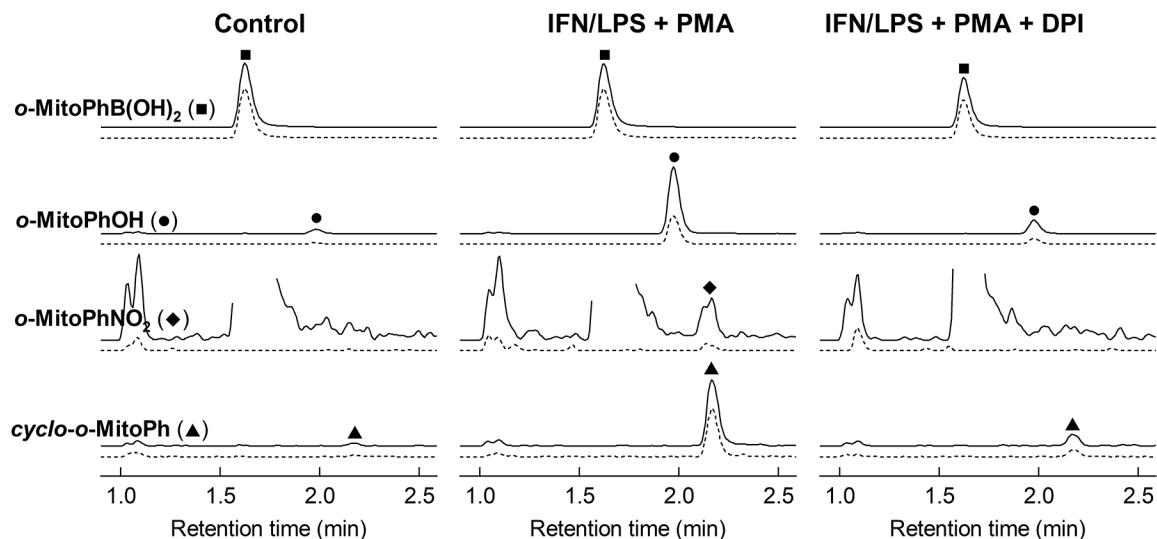
**Figure 1.** Scheme of oxidation of *o*-MitoPhB(OH)<sub>2</sub>.

*o*-MitoPhB(OH)<sub>2</sub> is oxidized to *o*-MitoPhOH by both ONOO<sup>-</sup> and H<sub>2</sub>O<sub>2</sub>. However, *cyclo-o*-MitoPh and *o*-MitoPhNO<sub>2</sub> are formed only in reaction between *o*-MitoPhB(OH)<sub>2</sub> and ONOO<sup>-</sup> as the minor products, with the yields of 10.5% and 0.5%, respectively (21).



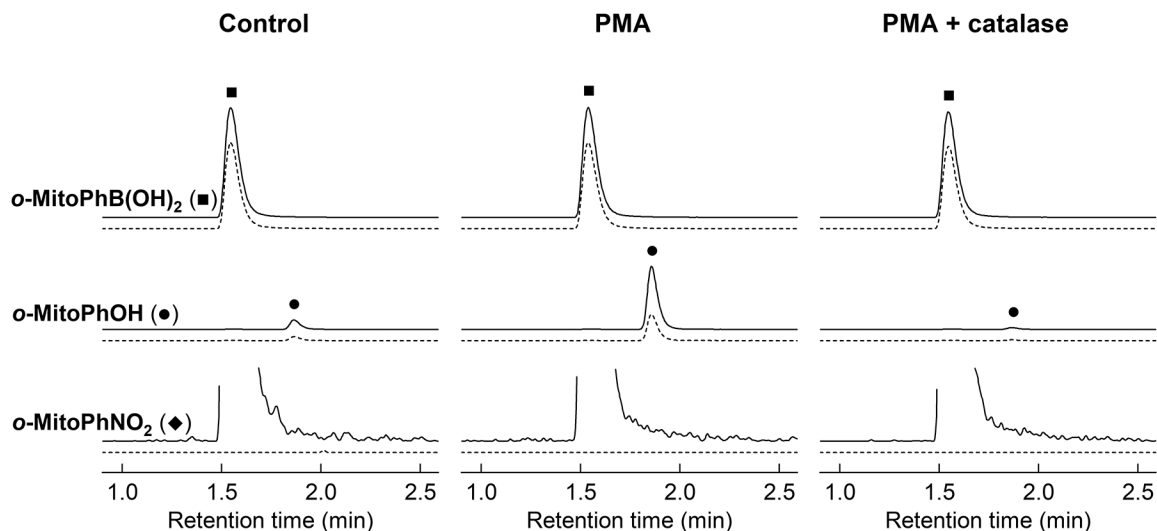
**Figure 2. HPLC-MS/MS detection of *o*-MitoPhB(OH)<sub>2</sub>, *o*-MitoPhOH, *cyclo-o*-MitoPh and *o*-MitoPhNO<sub>2</sub>.**

The chromatograms have been obtained with the described method by injection of 20  $\mu$ l of the mixture of *o*-MitoPhB(OH)<sub>2</sub>, *o*-MitoPhOH, *cyclo-o*-MitoPh and *o*-MitoPhNO<sub>2</sub> (solid symbols) and corresponding internal standards (open symbols) (1  $\mu$ M each). For each compound two MRM transitions are shown: dominant (solid lines) and reference (dashed lines). HPLC-MS/MS traces were recorded using the parameters shown in Table 2. The traces have not been scaled and thus represent the actual intensities of each MRM transition in the equimolar mixture.



**Figure 3. HPLC-MS/MS analysis of the extracts of RAW 264.7 macrophages activated to produce peroxynitrite.**

To produce peroxynitrite RAW 264.7 cells were pretreated overnight with interferon  $\gamma$  (IFN, 50 U/ml) and lipopolysaccharide (LPS, 1  $\mu\text{g/ml}$ ) followed by addition of PMA (1  $\mu\text{M}$ ) in the presence or absence of diphenyleneiodonium (DPI, 1  $\mu\text{M}$ ). During stimulation with PMA, *o*-MitoPhB(OH)<sub>2</sub> (50  $\mu\text{M}$ ) was present and incubated for 1 h. Cell pellets were collected and processed as described in the protocol. The intensities of *o*-MitoPhOH were multiplied by a factor of 10 and intensities of *cyclo-o*-MitoPh and *o*-MitoPhNO<sub>2</sub> were multiplied by 200 and 2000, respectively, to fit the same scale as of *o*-MitoPhB(OH)<sub>2</sub>. Solid lines represent the dominant transitions used for quantification and the dashed lines represent the reference transitions used for confirmation of peak identity.



**Figure 4. HPLC-MS/MS analysis of the media of neutrophil-like cells activated to produce hydrogen peroxide.**

To produce hydrogen peroxide, HL60 cells differentiated for 4 days with *all-trans* retinoic acid were stimulated with PMA (1  $\mu$ M) in the presence and absence of catalase (1 kU/ml) and co-treated with *o*-MitoPhB(OH)<sub>2</sub>. Aliquots of media were collected after 30 min and processed and analyzed as described in the protocol. The intensities of *o*-MitoPhOH were multiplied by a factor of 5 and intensities of *o*-MitoPhNO<sub>2</sub> were multiplied by 1000, to fit the same scale as of *o*-MitoPhB(OH)<sub>2</sub>.

**Table 1.**

## HPLC method parameters

Flow rate:	0.5 ml/min		
Gradient:	0 min	75% A	25% B
	2 min	68.3% A	31.7% B
	4 min	0% A	100% B
	4.5 min	0% A	100% B
	5 min	75% A	25% B
Diverter valve:	0 min	waste	
	1.0 min	detector	
	4.0 min	waste	

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**Table 2.**

MS/MS detection parameters

Analyte	Dominant MRM transition	Reference MRM transition	Reference MRM to dominant MRM intensities ratio	Retention time (min)
<i>o</i> -MitoPhB(OH) <sub>2</sub>	397.00>135.00	397.00>379.05	0.8	1.61
<i>o</i> -MitoPhB(OH) <sub>2</sub> - <i>d</i> <sub>15</sub>	412.20>117.10	412.20>135.10	0.55	1.57
<i>o</i> -MitoPhOH	369.00>107.10	369.00>183.05	0.4	1.95
<i>o</i> -MitoPhOH- <i>d</i> <sub>15</sub>	384.10>278.10	384.10>107.10	1.0	1.90
<i>o</i> -MitoPhNO <sub>2</sub>	397.90>262.05	397.90>351.10	0.25	2.13
<i>o</i> -MitoPhNO <sub>2</sub> - <i>d</i> <sub>15</sub>	413.10>277.15	413.10>113.00	0.85	2.08
<i>Cyclo-o</i> -MitoPh	351.10>183.05	351.10>165.05	0.7	2.15
<i>o</i> -MitoPhCH <sub>3</sub>	367.00>105.05	367.00>79.05	0.1	2.78