Activity-based fluorescent reporters for monoamine oxidases in living cells†

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Monoamine Oxidase Reporters 1 and 2 (MR1 and MR2) are substrate-tethered activity probes that utilize a tandem amine oxidation/β-elimination mechanism to detect MAO chemistry *in vitro* and in living cells directly and specifically without the need for additional enzymes or other activating reagents.

Monoamine oxidases (MAOs) are essential redox enzymes in the body that help maintain the proper balance of neurotransmitters and other dietary and biogenic amines.[1] MAOs are of particular importance to the brain and central nervous system, as their activity is critical for neural development and homeostasis; in addition, their genetically determined levels may contribute to aspects of personality and addictive behavior.[1,2] On the other hand, altered levels of MAO activity are associated with many neurodegenerative and psychiatric disorders arising from situations of uncontrolled neurotransmitter homeostasis and/or oxidative stress.[1–3] Because of their far-ranging impacts on neurological health and disease, MAOs are key therapeutic targets for a variety of brain disorders.[4] In particular, MAO inhibitors were among the first drugs discovered for treating clinical depression and are currently being explored as therapeutics for many brain afflictions, including Parkinson's disease, Alzheimer's disease, and cerebral ischemia during strokes.[4,5]

At the cellular and molecular level, MAOs are localized to the outer membrane of mitochondria and catalyze the aerobic, oxidative deamination of a variety of natural amine substrates, including serotonin, histamine, and the catecholamines dopamine, noradrenaline, and adrenaline.[1] These proteins utilize a flavin redox cofactor and are found in two isoforms, MAO A and MAO B, which are encoded by two distinct genes.[1,6] In the brain, MAO A is predominantly expressed in catecholaminergic neurons whereas MAO B is expressed largely in supporting glial cells.[1] The overall chemical reaction mediated by MAO generates the corresponding aldehyde and either ammonia or a substituted amine from the original amine substrate. Notably, the MAO reaction also produces an equivalent of hydrogen peroxide (H_2O_2) per turnover, a reactive oxygen species that can have diverse physiological or pathological effects in the cell. H_2O_2 formed during MAO activity may have metabolic and signaling functions,[7] but uncontrolled peroxide production can lead to oxidative stress and damage events connected to aging and neurodegenerative disease.[4,8] In the context of our programmatic interest in complex roles of H_2O_2 in cellular systems,[9] the production of aldehydes, amines, and H_2O_2 by a single enzymatic reaction underscores the potential of MAO chemistry to influence a variety of cellular pathways in parallel.

The contributions of MAOs to oxidation biology in various stages of health and disease, particularly in the brain, have prompted interest in devising selective and sensitive ways to monitor their function in living biological systems. To this end, a number of interesting fluorimetric[10] and colorimetric[11] approaches have been described for assaying MAO activity *in vitro*. Despite these advances, there are no reports of specific activity-based MAO probes for use within living cells and more complex natural environments. Available probes are limited for such applications owing to the need for a secondary activating enzyme that must be introduced externally to the system, indirect monitoring of products that can be generated by action of other cellular proteins (*e.g.* H_2O_2), and/or ultraviolet excitation that can damage or trigger background autofluorescence from cellular samples. In this report, we present a new class of activity-based fluorescent probes for MAOs and their application for detecting MAO chemistry in living cells. Monoamine Oxidase Reporters 1 and 2 (MR1 and MR2) are substrate-based reporters that offer facile, direct monitoring of MAO activity in aqueous solution and in living cells without the need for additional proteins or activating reagents.

Our general strategy for MAO activity detection is based on a tandem amine oxidation/β-elimination mechanism. We chose resorufin as a fluorescent reporter owing to its favorable photophysical properties and biological compatibility. In addition, *O*-alkylated resorufin derivatives are virtually non-fluorescent,[12] providing a molecular switch for activity detection. By using a propyl linker between the amine substrate and fluorophore portions of the probe, we reasoned that amine oxidation by MAO would afford iminium or aldehyde intermediates that would readily undergo β-elimination in water, generating acrolein and red-fluorescent resorufin as products. Our approach offers the distinct advantages of monitoring MAO chemistry directly and specifically without the need for an additional secondary activating enzyme.

Scheme 1 outlines the syntheses and MAO activations of MR1 and MR2 based on this oxidation/β-elimination design. Reactions of *N*-methyl-3-chloropropylamine or 3-chloropropylamine with Boc_2O furnish the protected amines **1** and **2** in 63% and 87% yield, respectively. Alkylations of **1** and **2** with the sodium salt of resorufin afford dyes **3** and **4** in 55% and 56% yield, respectively. Deprotections of **3** and **4** with TFA provide the final MR1 (**5**) and MR2 (**6**) probes, respectively, in near quantitative yield. The dyes

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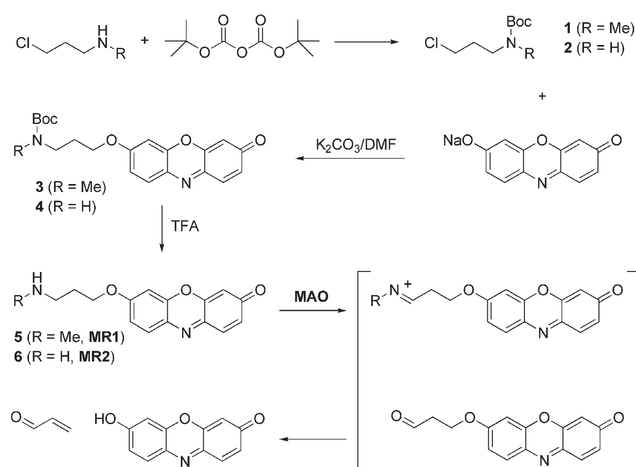

Scheme 1 Monoamine Oxidase Reporters 1 and 2 (MR1/MR2).

were identified and then characterized by ^{1}H NMR and mass spectrometry. Notably, the short and convergent reaction sequences afford a wide range of potential activity probe candidates by varying combinations of substrate amine head groups and *O*-alkylated fluorophores.

Spectroscopic evaluations of MR1 and MR2 were carried out under simulated physiological conditions (phosphate-buffered saline, PBS, pH 7.4). The absorption spectrum of MR1 features one prominent band in the visible region centered at 480 nm ($\varepsilon = 1.3 \times 10^4$ M^{-1} cm^{-1}) with a shoulder at 404 nm ($\varepsilon = 6.5 \times 10^3$ M^{-1} cm^{-1}). The spectrum of MR2 has a similar profile (λ_{abs} = 480 nm, $\varepsilon = 1.5 \times 10^4$ M^{-1} cm^{-1}; λ_{abs} = 406 nm, $\varepsilon = 7.9 \times 10^3$ M^{-1} cm^{-1}). As expected, both *O*-alkylated dyes are almost non-fluorescent before MAO oxidation (λ_{em} = 584 nm, $\Phi < 0.01$ for MR1; λ_{em} = 583 nm, $\Phi < 0.01$ for MR2) compared to resorufin ($\Phi = 0.6$). Furthermore, both reagents remain stable in aerated, aqueous buffer for days at room temperature.

We next assessed the *in vitro* reactivity of MR1 and MR2 with MAO A and MAO B, the two major isoforms of this enzyme. To our delight, both probes are competent substrates for each enzyme and afford direct fluorescent readouts of activity. Absorption and emission measurements, along with electrospray mass spectrometry, confirm that resorufin is the product obtained upon reactions of MR1 or MR2 with MAO A or B. The activity-based fluorescent probes were assayed over a wide range of dye concentrations (0 to 250 μM) with both MAO isoforms at low concentrations (10 μg mL^{-1}). A representative enzyme kinetics plot is given in Fig. 1a. These kinetics experiments afford the following Michaelis–Menten constant values for MR1 and MR2 with MAO A and MAO B: K_m MR1/MAO A = 7.6 ± 1.0 μM, K_m MR1/MAO B = 1.8 ± 0.2 μM, K_m MR2/MAO A = 6.3 ± 0.6 μM, and K_m MR2/MAO B = 3.4 ± 0.5 μM. The K_m values obtained for the MR probes are lower than the μM to mM K_m range observed for reactions of MAO A and B with natural amine substrates. At the K_m values for each MR substrate, the maximum fluorescence enhancements for MR1 and MR2 activity probes to their resorufin products are 58-fold and 275-fold for MAO A and 71-fold and 286-fold for MAO B, respectively. The large turn-on responses for the MR activity probes are due to the sizeable dynamic range of the bright resorufin dye platform as well as the relatively fast kinetics of their reactions with the MAO isoforms. Fig. 1b displays a representative fluorescence response of MR1 to MAO A.

With spectroscopic characterization of MR1 and MR2 in hand as well as *in vitro* results showing the kinetic competence of these small-molecule reporters for direct detection of MAO activity, we turned our attention to applying these fluorescent probes in living cells. We chose the PC12 cell line for these experiments owing to its relatively high endogenous expression of MAO and its neuron-like characteristics in culture when supplemented with nerve growth factor (NGF). Live PC12 cells were grown with 30 ng mL^{-1} NGF to trigger process formation and loaded with 250 μM MR1 or MR2. The MR-loaded cells were then incubated for up to 7 h at 37 °C, washed thoroughly to remove extracellular reporter dye, resuspended in PBS buffer (pH 7.4), and assayed directly using fluorescence spectroscopy. The emission data collected in Fig. 2 show detectable increases in accumulated MAO activity in live

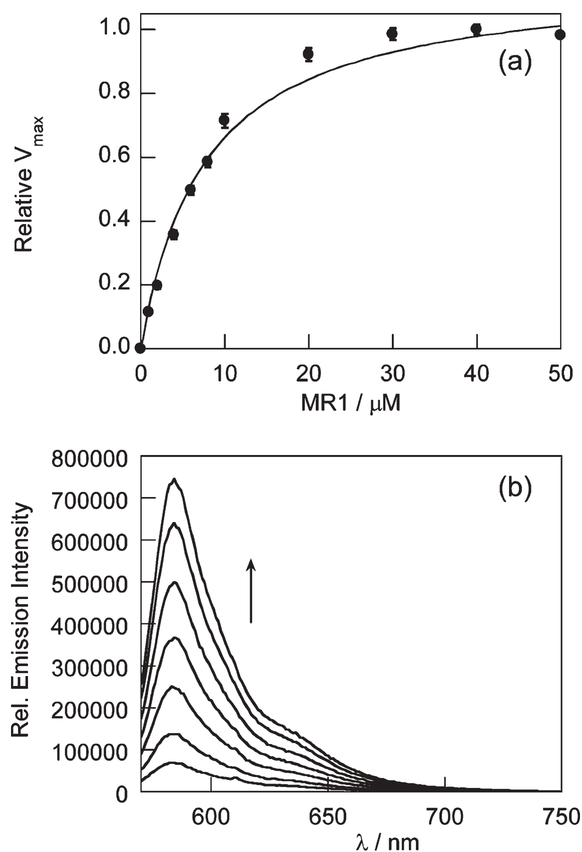

Fig. 1 Direct fluorimetric detection of MAO activity with MR1. (a) Enzyme kinetics plot of reactions of MR1 with MAO A. Data show relative reaction rates measured over a range of MR1 concentrations, and each data point represents the average of at least six independent experiments. Reactions were performed at 25 °C in enzyme assay buffer (100 mM HEPES, pH 7.4 with 5% glycerol and 1% DMSO) with MAO A at a final protein concentration of 10 μg mL^{-1}. Fluorescence excitation was provided at 544 nm and emission intensity collected at 590 nm. (b) Representative fluorescence response of MR1 to 50 μg mL^{-1} MAO A at its K_m value (7.6 μM probe). Reactions were performed at 25 °C in enzyme assay buffer (100 mM HEPES, pH 7.4 with 5% glycerol and 1% DMSO). Fluorescence excitation was provided at 550 nm, and traces are shown after 0, 10, 20, 30, 40, 50, and 60 min after adding MR1 to the MAO enzyme. Deprotections of MR1 with MAO A under these conditions are not complete at these early time points.

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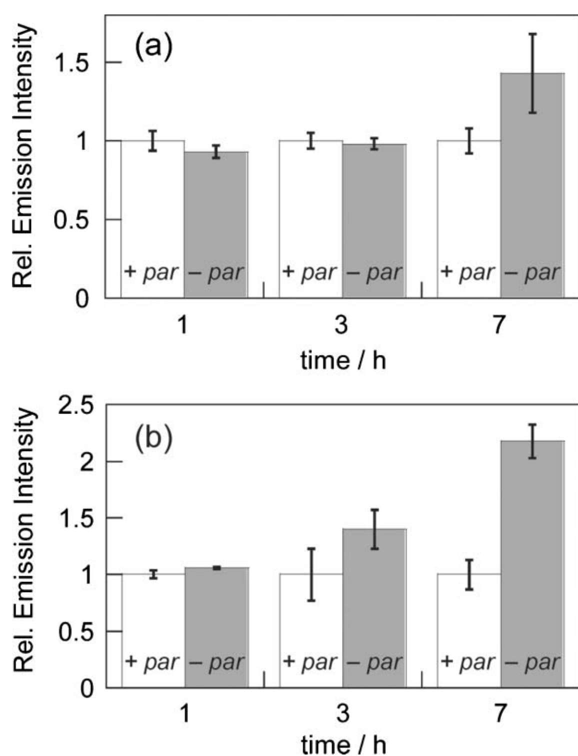

Fig. 2 Direct fluorescence detection of MAO activity in live neuron-like cells with MR1 and MR2. Live PC12 cells were loaded with 250 μM MR1 (panel a) or MR2 (panel b) for up to 7 h at 37 °C, with or without pre-treatment with the general MAO inhibitor pargyline (250 μM, 80 min, 37 °C). Fluorescence responses of the activity-based MR probes are normalized relative to intensities obtained for pargyline-inhibited samples. White bars represent data from cells pre-treated with pargyline before the MR probe, and gray bars show data from cells treated with MR probe only.

PC12 cells over this time period using MR1 or MR2. Moreover, these MAO-dependent turn-on responses can be ameliorated by pre-treatment with the general MAO inhibitor pargyline (250 μM, 80 min, 37 °C).[4] Control experiments show that cells without MR dye give no background fluorescence and that the pargyline inhibitor does not react with either MR1 or MR2. In addition, the cells are viable throughout the experiments as shown by brightfield transmission measurements. The data establish that MR1 and MR2 are a unique set of small-molecule chemical tools that can directly and specifically report MAO activity in living cells by fluorescence.

In closing, we have described two small-molecule reporters that afford direct fluorimetric detection of MAO activity in aqueous solution and in living cells. The substrate-based probes MR1 and MR2 feature large turn-on fluorescence enhancements upon reaction with either MAO A or B, as well as visible wavelength excitation and emission profiles to minimize biosample photo-damage and interfering autofluorescence. *In vitro* experiments with purified MAO A and B enzymes show that both MR1 and MR2 are kinetically competent to report activity of these enzymes, with low K_m values comparable with natural amine substrates found in cells. Moreover, the MR reagents are membrane-permeable and can be used to report MAO activity within living cells without the need for gene transfection or addition of any other reagents. Taken together, these results establish the potential utility of MR1 and MR2 for biological applications and provide a starting point for designing a chemical toolbox for assaying MAO function in living systems. We are currently exploring opportunities for MR1, MR2, and related new reagents for probing the chemistry and chemical biology of MAO in complex natural settings, with particular interest in live-cell models of neurodegenerative disease.

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