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Fluorescent probes for detecting monoamine oxidase activity and cell imaging†

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Xuefeng Li,[a] Huatang Zhang,[b] Yusheng Xie,[b] Yi Hu,[c] Hongyan Sun*[b] and Qing Zhu*[a]

A series of new fluorogenic probes for monoamine oxidases (MAOs) were reported based on an oxidation and β-elimination mechanism. The limits of detection of the probes for MAO-A and -B were determined to be 3.5 and 6.0 μg mL^{-1} respectively. These probes displayed strong activity towards MAOs, especially MAO-B. Cellular imaging studies were also successfully conducted with MCF-7 cells.

Monoamine oxidases (MAOs) are flavin adenine dinucleotide (FAD)-containing enzymes that catalyze the α-carbon oxidation of amine neurotransmitters in human brains.[1] MAOs are encoded by two distinct genes and expressed as two isozymes, MAO-A and MAO-B. Despite their substantial similarities, these two isozymes differ in many aspects, including substrate specificities, tissue distribution and sensitivity to inhibitors.[2] For example, MAO-A mainly metabolizes serotonin and norepinephrine, and MAO-B preferentially breaks down benzylamine and phenylethylamine. Notwithstanding, tyramine and dopamine can be metabolized to their corresponding aldehydes by both isoforms.[3] Recent studies have shown that MAOs are closely linked to the increasing concentration of amines in neurons and are responsible for various types of adverse health conditions, such as depression, senility, Alzheimer's disease, Parkinson's disease and schizophrenia.[4] Due to MAOs' important roles in these diseases, strong interest has been generated in the research community to develop various types of assays to detect MAO activity.

Among the various approaches, fluorescence spectroscopy methods for detecting MAOs have attracted much attention due to their high sensitivity and simplicity.[5] The previously developed methods all reveal good specificity toward MAO-B, whereas few of them have reported the limit of detection (LOD) of the assays.[6] Zhu *et al.*, for example, synthesized two simple coumarin probes, which exhibited good sensitivity towards MAOs.[7] In another study, two resorufin probes were designed based on an amine oxidation/β-elimination mechanism. Significant fluorescence enhancements can be observed upon reaction with MAOs.[8] However, coumarin's excitation wavelength, which is near the ultraviolet region, restricts its usage in cellular imaging studies. The high price of resorufin also prevents the probe from being widely adopted, and moreover, no cell imaging studies were conducted for this probe. It is therefore imperative to develop novel fluorescent probes for detecting MAOs in cells. The improved fluorescent properties and low cost will allow the probe to be widely adopted in biological applications.

In this study, we designed and synthesized a series of novel activity-based fluorescent probes to detect MAOs. Our design of the probes is based on the fluorescein scaffold, which belongs to the family of xanthene dyes and has become a prevalent fluorophore in fluorescence spectroscopy studies due to its excellent spectral properties.[9] Fluorescein-based probes have been widely used to detect activities of various classes of enzymes.[10] We noticed that, when the two phenolic OH groups of a fluorescein derivative are alkylated, the derivative is non-fluorescent, and this property provides a molecular off–on switch for enzymatic activity-based detection.[11] Therefore, in our design, one of the hydroxyl groups is linked to an aminopropoxy group, which is the substrate of MAOs, and the other is connected to different functional groups to attain high selectivity and excellent cell-permeability as reported in our previous paper.[12]

Our design is shown in Scheme 1; the 3-aminopropoxy group, a substrate of MAOs, is installed on one phenolic OH of fluorescein. Under the catalysis of MAOs, the propylamino

[a]*Institute of Bioengineering, Zhejiang University of Technology, Chaowang Road 18, Hangzhou 310014, China. E-mail: zhuq@zjut.edu.cn; Fax: +86-571-88320781; Tel: +86-571-88320781*

[b]*Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong, China. E-mail: hongysun@cityu.edu.hk*

[c]*CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, CAS Key Lab of Nuclear Radiation and Nuclear Energy Technology, Center for Multidisciplinary Research, Institute of High Energy Physics, Chinese Academy of Sciences (CAS), Beijing 100049, China*

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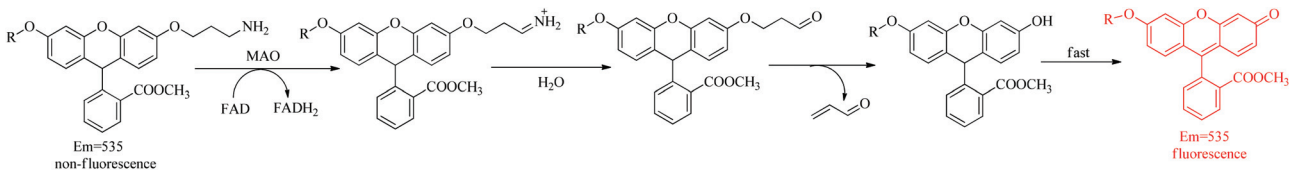
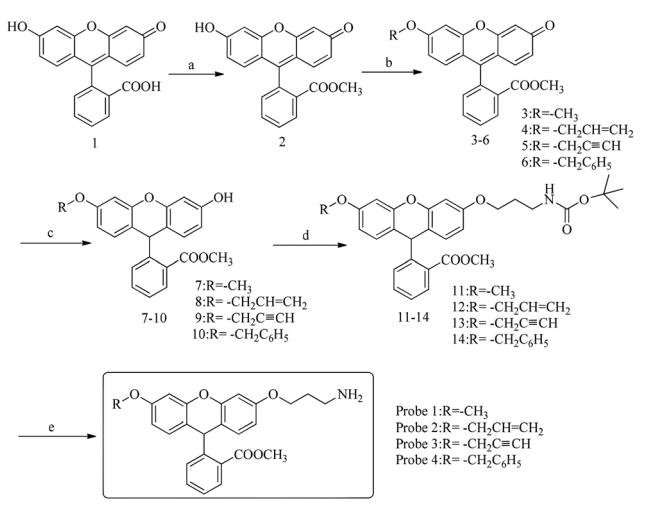


Scheme 1 Design principle of the fluorescent probes for MAO.

group will be oxidized to the corresponding imine, which will be subsequently hydrolyzed to yield the aldehyde. The resulting aldehyde derivative is unstable and will undergo β-elimination quickly, leading to the breakage of the ether bond. The intermediate is quickly air-oxidized to afford fluorescein, and fluorescence can therefore be turned on in the presence of MAOs.[13]

Based on the above hypothesis, we designed and synthesized four different fluorescent probes. In the specific probe design, one phenolic OH group of fluorescein was substituted with the 3-aminopropoxy group. The other group was replaced with various functional groups to improve the specificity and hydrophobicity of the probes. Scheme 2 outlined the synthetic route of probes 1–4. First, fluorescein **1** was converted to methyl fluorescein **2** in 72.3% yield. Fluorescein **2** was then reacted with different electrophilic reagents, namely iodomethane, allyl bromide, propargyl bromide and benzyl bromide, to obtain compounds **3–6** respectively. Subsequent reduction in a solution of $NaBH_4$ in MeOH gave intermediates **7–10** with good yields. *N*-Boc-3-bromepropylamine was then added to the corresponding intermediates, and compounds **11–14** were obtained. Finally the Boc group was removed by $ZnCl_2$ and the desired probes were obtained with moderate yields after the hydrolysis in 2 M NaOH. The compounds were purified and characterized by ^1H (or ^{13}C)-NMR and mass spectrometry.

Next we carried out MAO assays with fluorescein probes 1–4. Preliminary experiments showed that all the probes *per se* displayed virtually no fluorescence, and the fluorescence intensity increased to a steady state around 40 min after the addition of MAOs. We optimized the assay conditions as the following: a fluorescent probe (200 μM) and an enzyme solution (16 μg mL^{-1}) were mixed with 200 μL boric acid buffer (pH = 8.4) in 96-well plates at 37 °C for 40 minutes. All fluorescence spectra were collected at $\lambda_{ex}/\lambda_{em}$ = 470/535 nm with a microplate reader. In general, no change in fluorescence was observed in the absence of MAO, whereas the fluorescence intensity increased immediately upon addition of MAO enzymes (Fig. 1a). The results indicated that all four probes can be used to monitor the activity of MAOs. It is also noted



Scheme 2 Synthesis of fluorogenic probes for monoamine oxidase: (a) H_2SO_4–MeOH, r.t.; (b) iodomethane, allyl bromide, propargyl bromide or benzyl bromide, K_2CO_3 in DMF, r.t.; (c) $NaBH_4$ in MeOH, 0 °C; (d) $Br(CH_2)_3NHBoc$, K_2CO_3 in DMF, r.t.; (e) $ZnCl_2$–CH_2Cl_2, r.t.

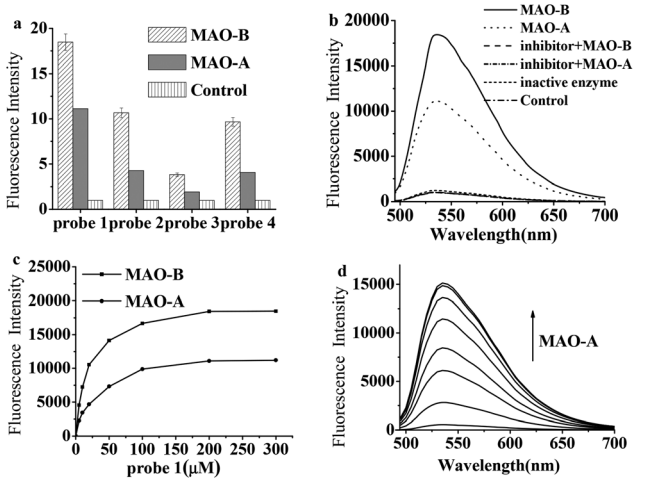


Fig. 1 (a) Fluorescence intensity of probes 1–4 before and after reactions with MAO-B or MAO-A. The bars represent the fluorescence intensity of the probes (200 μM) after 40 minutes at $\lambda_{ex}/\lambda_{em}$ = 470/535 nm in 100 mM aqueous borate buffer (pH = 8.4). The data were derived from the means of three independent experiments, and the error bars represent the standard deviation. (b) Fluorescence intensity of probe 1 before and after reaction with MAO-B (solid line), MAO-A (dot line) and the corresponding inhibitor and inactive enzyme. The spectra were recorded in 100 mM borate buffer (pH = 8.4) at λ_{ex} = 470 nm. (c) Data showing the activity of different concentrations of probe 1 (0–300 μM) after reaction with MAOs (16 μg mL^{-1}) at 37 °C in enzyme assay buffer (100 mM borate buffer, pH = 8.4). The fluorescence intensity was monitored at $\lambda_{ex}/\lambda_{em}$ = 470/535 nm. (d) Fluorescence of probe 1 (200 μM) treated with different concentrations of MAO-A, [enzyme] = 0–0.3 mg mL^{-1} after 40 minutes in pH = 8.4 borate buffer.

that all the probes displayed higher reactivity towards MAO-B, compared to MAO-A. The signal-to-background ratios of the four probes for MAO-B were 18.5, 10.7, 3.8, and 9.7, and those for MAO-A were 11.1, 4.3, 1.9 and 4.1, respectively. The LOD were 3.5 and 6.0 μg mL^{-1} for MAO-A and -B respectively by setting the signal to noise ratio as 3 : 1,[14] which was comparable with the commercially available assay kit.[6] Among the four probes, probe 1 showed the highest reactivity toward MAOs. Consequently, it was chosen for further biological experiments. Further data analysis showed a strong fluorescence signal at an emission band of 535 nm with the addition of the MAOs (Fig. 1b and Fig. S1, ESI†). The fluorescence intensity of probe 1 with MAO-B was found to be about twice as high as that of probe 1 with MAO-A. Very little fluorescence increase was detected when the enzymes were denatured or inhibited with the corresponding inhibitors, further confirming that the fluorescence intensity correlates with MAO activity. As shown in Fig. 1c, the fluorescence intensity increases with increasing concentrations of probe 1. A near linear relationship can be observed between fluorescence intensity and concentration in the low concentration range. Fig. 1d shows a fluorescence spectrum of probe 1 with the addition of various concentrations of MAO-A. Taken together, these studies demonstrate that probes 1–4 can be used to monitor the activity of MAOs.

Subsequently, we carried out a more detailed kinetic study by analyzing the reactions of the four probes in an extensive range of concentrations with MAO-A and MAO-B. The concentration-dependent data of probes 1–4 were collected and fitted to a Lineweaver–Burk plot. The Michaelis–Menten constant was then derived from the plot and is summarized in Table 1. Results revealed that all K_m values of MAO-A are slightly higher than those of MAO-B, indicating that all the probes have higher reactivity towards MAO-B. The catalytic efficiency (k_{cat}/K_m) of MAOS is higher for probe 1 than for the other three probes, suggesting that probe 1 has a better reactivity towards MAOs. It was noted that our probes produced similar K_m values as the results of Chang's group and they are much lower than dopamine, a physiological substrate of MAOs. This proves that it is reasonable to replace resorufin with fluorescein to detect MAO activity. This study further indicated that probe 1 with a high k_{cat} value would be the preferable probe for assaying MAOs.

A fluorogenic probe is a powerful tool for cell imaging studies due to its high signal-to-noise ratio. Thus, after studying the kinetics of the probes in great detail, we went further to examine whether our probes are suitable for a cell imaging study, which was not done in the previous report.[7] We chose MCF-7 cells (human breast carcinoma cells) because they are human MAO positive cells. Specifically, MCF-7 cells were seeded at a density of 1.0×10^6 cells per well in a 6-well tissue culture dish overnight. The cells were incubated with probe 1 in a 5% CO_2 incubator at 37 °C for 3 hours. The medium was then removed, and the cells were washed twice with phosphate buffered saline (PBS, pH = 7.4) to completely remove excess extracellular probes. For control experiments, the cells were treated with a MAO-B inhibitor, selegiline, for 60 minutes at 37 °C prior to the incubation with probe 1 (50 μM) for 2 hours. Subsequently, the cells were imaged using a fluorescence microscope (Fig. 2). Moreover, addition of the MAO-B inhibitor significantly suppressed the fluorescence signals arising from the breakdown of probe 1. Our experiments with probe 1 and live MCF-7 cells further suggested that the probes reported in our study can serve as promising small-molecule tools which directly report MAO's activity in living cells.

In conclusion, we have successfully designed and synthesized four novel off–on switch fluorescent probes. Compared with previously reported fluorescent probes, these new probes display stronger activity towards MAOs and possess improved fluorescent properties. Furthermore, cell imaging

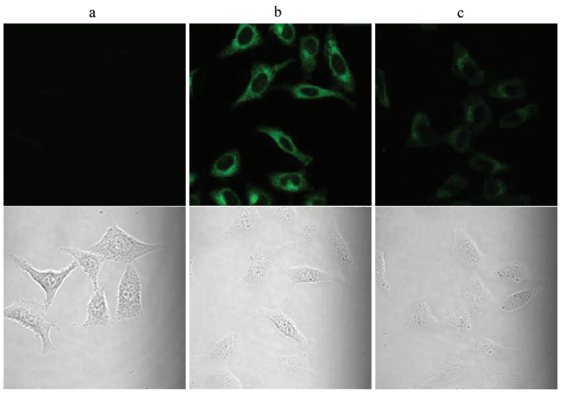


Fig. 2 Fluorescence images of MCF-7 cells with probe 1. Fluorescence images and bright-field of (a) MCF-7 cells, (b) MCF-7 cells with probe 1 at 50 μM, and (c) MCF-7 cells pretreated with the inhibitor selegiline and then incubated with probe 1 at 50 μM.

Table 1 The kinetic constants of probes 1–4 with MAOs

	K_m (μM)		k_{cat} (min^{-1})		k_{cat}/K_m (M^{-1} min^{-1})	
Probe	MAO-A	MAO-B	MAO-A	MAO-B	MAO-A	MAO-B
1	11.4	7.7	3.0	3.5	263.2×10^3	454.5×10^3
2	8.9	6.8	1.5	1.9	168.5×10^3	279.4×10^3
3	20.6	11.4	0.6	0.7	29.1×10^3	61.4×10^3
4	11.4	10.5	0.3	0.4	26.3×10^3	38.1×10^3
MR2[7]	6.3	3.4	—	—	—	—
Dopamine	—	93	—	—	—	—

studies also proved that the probes are suitable for monitoring the activity of MAOs in living cells.

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Notes and references

1 (*a*) A. I. Meyers and W. T. Hoeve, *J. Am. Chem. Soc.*, 1980, **102**, 7126; (*b*) J. R. Miller and D. E. Edmondson, *Biochemistry*, 1999, **38**, 13670; (*c*) W. Shen, S. B. Long, S. A. Yu, W. W. Chen and Q. Zhu, *Med. Chem. Res.*, 2012, **21**, 3858.

2 Y. X. Zhang, Y. F. Xu, S. Y. Tan, L. Xu and X. H. Qian, *Tetrahedron Lett.*, 2012, **53**, 6881.

3 (*a*) J. S. Fowler, J. Logan, N. D. Volkow, G. J. Wang, R. R. MacGregor and Y. S. Ding, *Methods*, 2002, **27**, 263; (*b*) C. C. Wang, E. Billett, A. Borchert, H. Kuhn and C. Ufer, *Cell. Mol. Life Sci.*, 2013, **70**, 599.

4 (*a*) R. B. Silverm, *Acc. Chem. Res.*, 1995, **28**, 335; (*b*) M. J. Zhou and N. Panchuk-Voloshina, *Anal. Biochem.*, 1997, **253**, 169; (*c*) W. H. Zhou, M. P. Valley, J. Shultz, E. M. Hawkins, L. Bernad, T. Good, D. Good, T. L. Riss, D. H. Klaubert and K. V. Wood, *J. Am. Chem. Soc.*, 2006, **128**, 3122; (*d*) M. P. Valley, W. H. Zhou, E. M. Hawkins, J. Shultz, J. J. Cali, T. Worzella, L. Bernad, T. Good, D. Good, T. L. Riss, D. H. Klaubert and K. V. Wood, *Anal. Biochem.*, 2006, **359**, 238; (*e*) H. R. Park, J. Kim, T. Kim, S. Jo, M. Yeom, B. Moon, H. Choo, J. Lee, E. J. Lim, K. D. Park, S. J. Min, G. Nama, G. Keuma, C. J. Lee and H. Choo, *Bioorg. Med. Chem.*, 2013, **21**, 5480; (*f*) S. Banerjee and M. K. Poddar, *J. Physiol. Sci.*, 2013, **63**, 279.

5 (*a*) G. Chen, D. J. Yee, N. G. Gubernator and D. Sames, *J. Am. Chem. Soc.*, 2005, **127**, 4544; (*b*) J. X. Aw, Q. Shao, Y. M. Yang, T. T. Jiang, C. Y. Ang and B. G. Xing, *Chem. Asian J.*, 2010, **5**, 1317.

6 M. P. Valley, E. M. Hawkins, J. Shultz, *et al.*, *Cell Notes*, 2006, **14**, 4.

7 (*a*) Y. Y. Lu, Y. G. Wang, B. Dai, Y. Q. Dai, Z. Wang, Z. W. Fu and Q. Zhu, *Chin. Chem. Lett.*, 2008, **19**, 947; (*b*) S. B. Long, L. Chen, Y. M. Xiang, M. G. Song, Y. G. Zheng and Q. Zhu, *Chem. Commun.*, 2012, **48**, 7164.

8 A. E. Albers, K. A. Rawls and C. J. Chang, *Chem. Commun.*, 2007, 4647.

9 L. D. Lavis, T. J. Rutkoski and R. T. Raines, *Anal. Chem.*, 2007, **79**, 6775.

10 (*a*) K. Tanaka, T. Miura, N. Umezawa, Y. Urano, K. Kikuchi, T. Higuchi and T. Nagano, *J. Am. Chem. Soc.*, 2001, **123**, 2530; (*b*) Y. Urano, M. Kamiya, K. Kanda, T. Ueno, K. Hirose and T. Nagano, *J. Am. Chem. Soc.*, 2005, **127**, 4888; (*c*) L. F. Mottram, E. Maddox, M. Schwab, F. Beaufils and B. R. Peterson, *Org. Lett.*, 2007, **9**, 3741.

11 (*a*) P. G. Mattingly, *Bioconjugate Chem.*, 1992, **3**, 430; (*b*) Y. S. Duan, M. Liu, W. Sun, M. Wang, S. Z. Liu and Q. X. Li, *Mini-Rev. Org. Chem.*, 2009, **6**, 35.

12 Y. M. Xiang, B. Y. He, X. F. Li and Q. Zhu, *RSC Adv.*, 2013, **3**, 4876.

13 (*a*) D. Wahler, F. Badalassi, P. Crotti and J.-L. Reymond, *Angew. Chem., Int. Ed.*, 2001, **40**, 4457; (*b*) Z. Shi, X. Tang, X. Zhou, J. Cheng, Q. Han, J. A. Zhou, B. Wang, Y. Yang, W. Liu and D. Bai, *Inorg. Chem.*, 2013, **52**, 12668.

14 H. Zhang, P. Wang, Y. Yang and H. Sun, *Chem. Commun.*, 2012, **48**, 10672.

