Article

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Highly Selective Fluorescent Probe Based on Hydroxylation of Phenylboronic Acid Pinacol Ester for Detection of Tyrosinase in Cells

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ABSTRACT

The detection of tyrosinase, a biomarker for melanoma, is of great significance. Herein, a fluorescent tyrosinase probe, with resorufin as the fluorophore and *m*-tolylboronic acid pinacol ester as the receptor, is proposed. The response relies on tyrosinase-catalyzed hydroxylation of phenylboronic acid pinacol ester at adjacent position followed by 1,6-rearrangement-elimination to release resorufin. This probe well quantifies tyrosinase in the range from 1 to 100 U mL^{-1} with detection limit of 0.5 U mL^{-1}. Importantly, the probe exhibits high selectivity for tyrosinase over other biological substances including reactive oxygen species. In addition, it is successfully applied to imaging of tyrosinase in cells. This probe provides a novel platform for selective detection of tyrosinase in biosystem.

KEYWORDS: Fluorescent Probe; Tyrosinase; Hydroxylation; Phenylboronic Acid Pinacol Ester

INTRODUCTION

In mammals, melanocytes as melanin-producing cells are predominantly localized in skin, hair and eyes.[1,2] The malignant mutation of melanocytes results in a kind of skin cancer called melanoma. Tyrosinase (EC 1.14.18.1), a key enzyme in melanogenesis, catalyzes the hydroxylation of monophenols to *o*-diphenols and the subsequent oxidation of *o*-diphenols to *o*-quinones.[3,4] Tyrosinase is a biomarker for melanoma, because its expression is mainly limited to melanocytes and its level is up-regulated during malignant mutation.[5–8] Therefore, the detection of tyrosinase in biosystem is of great importance.

Fluorescent probes have drawn much attention to monitor tyrosinase in biosystem due to their high sensitivity, simplicity and noninvasion. Phenols, natural substrate of tyrosinase, are usually designed as the receptors of fluorescent tyrosinase probes.[9–16] Most fluorescent tyrosinase probes are based on tyrosinase-catalyzed oxidation of phenols to quinones,[9–14] but their selectivity is not perfect and show response to reactive oxygen species (ROS), because phenols are also oxidized to quinones by ROS.[17–20] Considering that ROS do not have the function of hydroxylation, fluorescent tyrosinase probes based on tyrosinase-catalyzed hydroxylation of 3-methylphenol at adjacent position have been reported recently,[15,16] and they exhibit high selectivity and show no response to ROS. Given the above, highly selective fluorescent probes for tyrosinase are rare, and efforts are still needed.

In this work, fluorescent tyrosinase probe 1, synthesized via linking *m*-tolylboronic acid pinacol ester to resorufin, is developed for the detection of tyrosinase in cells. Probe 1 exhibits high selectivity and shows no response to ROS. As shown in Figure 1, phenylboronic acid pinacol ester in probe 1 is hydroxylated at adjacent position by tyrosinase, and then resorufin is released via 1,6-rearrangement-elimination. Resorufin is chosen as the fluorophore owing to its long wavelengths ($\lambda_{ex/em}$ = 550/583 nm), high fluorescence quantum yield (Φ = 0.75) and efficient fluorescence quenching via the alkylation of 7-hydroxy.[21–23]

EXPERIMENTAL SECTION

Reagents. 3-(Bromomethyl)phenylboronic acid, phenylboronic acid pinacol ester, 2-hydroxyphenylboronic acid pinacol ester, pinacol and resorufin were

purchased from Energy Chemical (Shanghai, China). Tyrosinase from mushroom, lipase from Candida rugosa and trypsin from bovine pancreas were obtained from Sigma-Aldrich (Shanghai, China). Catalase from bovine liver, glucose oxidase from Aspergillus niger, kojic acid, hydrogen peroxide, sodium hypochlorite, *tert*-butyl hydroperoxide (TBHP), potassium dioxide, glucose, vitamin C, vitamin B6, glycine, glutamic acid, cysteine, glutathione, tyrosine and creatinine were purchased from Aladdin Chemistry Co. Ltd (Shanghai, China). The water used throughout the experiments was supplied by Milli-Q system (Millipore, USA). Cell lines (B16 and HepG2) were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China). Other reagents were from qualified reagent suppliers. ROS including H_2O_2, TBHP, ClO^-, *tert*-butoxy radical (TBO·), HO·, $^1O_2$, $O_2^{\cdot-}$, $ONOO^-$ were prepared according to reported methods.[24,25] The stock solution of probe 1 (5.0 mM) was prepared in DMSO and stored at 4 °C in the dark.

Instrumentation. NMR spectra were measured with a JNM-ECS 400M Instrument (Japan). HRMS were measured using a Bruker Daltonics APEX II 47e FT-ICR Spectrometer (America). Fluorescence spectra were recorded on a RF-5301pc Fluorescence Spectrometer (Japan). Absorption spectra were measured on a TU-1810 UV-VIS Spectrophotometer (China). Fluorescence imaging was conducted on a DMI4000B Inverted Fluorescence Microscope (Germany).

Synthesis of Compound 1. 3-(Bromomethyl)phenylboronic acid (43 mg, 0.20 mmol) and pinacol (28 mg, 0.24 mmol) were dissolved in diethyl ether (15 mL). The solution was stirred overnight at room temperature, and then concentrated under

vacuum. The crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (2/1, v/v) as eluent. Compound 1 was obtained as white solid in 64% yield (38 mg). ^{1}H NMR (400 MHz, CDCl3): δ 7.82 (s, 1H), 7.74 (d, 1H), 7.50 (d, 1H), 7.36 (t, 1H), 4.51 (s, 2H), 1.35 (s, 12H). ^{13}C NMR (400 MHz, CDCl3): δ 137.18, 135.27, 134.87, 132.03, 128.33, 84.01, 33.55, 24.94. HRMS (ESI, *m*/*z*) Calcd. for [$C_{13}H_{18}BBrO_2 + H^+$]: 297.0656, found: 297.0655.

Synthesis of Probe 1. To a suspension of resorufin (26 mg, 0.12 mmol) in anhydrous DMF (10 mL), K_2CO_3 (25 mg, 0.18 mmol) and compound 1 (24 mg, 0.08 mmol) were added. After stirred overnight at room temperature, the mixture was diluted with ethyl acetate (50 mL) and then washed with saturated NaCl. The organic phase was collected and concentrated under vacuum. The crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (4/1, v/v) as eluent. Probe 1 was obtained as orange solid in 47% yield (16 mg). ^{1}H NMR (400 MHz, CDCl3): δ 7.88 (s, 1H), 7.82 (d, 1H), 7.71 (d, 1H), 7.55 (d, 1H), 7.45–7.41 (m, 2H), 7.02 (dd, 1H), 6.89 (d, 1H), 6.84 (dd, 1H), 6.33 (d, 1H), 5.17 (s, 2H), 1.36 (s, 12H). ^{13}C NMR (400 MHz, CDCl3): δ 186.41, 162.81, 149.91, 145.73, 145.70, 135.12, 134.79, 134.33, 134.07, 131.69, 130.61, 128.57, 128.37, 114.38, 106.84, 101.13, 84.13, 71.01, 24.98. HRMS (ESI, *m*/*z*) Calcd. for [$C_{25}H_{24}BNO_5 + H^+$]: 430.1820, found: 430.1819.

General Procedure for Spectra Measurement. PBS buffer (930 μL, 10 mM, pH 7.4), probe 1 (20 μL, 250 μM) and tyrosinase (50 μL) were placed into a 2.0 mL centrifugal tube. The solution was incubated at 37 □ for 5 h, and then its fluorescence

spectrum was recorded.

Cell Culture and Fluorescence Imaging. The two cell lines (B16 and HepG2) were cultured in RPMI 1640 medium supplemented with fetal bovine serum (10%), penicillin (100 U mL^{-1}) and streptomycin (100 U mL^{-1}) at 37 □ under 5% CO_2 environment. For fluorescence imaging, the adherent cells in a 6-well plate were incubated with probe 1 (2.5 μM) for 5 h and then washed with PBS three times. In inhibitor experiment, cells were pretreated with kojic acid (200 μM) for 2 h, then washed with PBS three times, and finally incubated with probe 1 (2.5 μM) for 5 h.

Cytotoxicity Assay. Cytotoxic effect of probe 1 was measured with colorimetric methyl thiazolyl tetrazolium (MTT) assay. B16 cells (1×10^4 cells/well) were seeded in a 96-well plate, then incubated for 24 h in the presence of probe 1 at different concentrations (0, 1.25, 2.5, 5, 7.5 μM), followed by adding MTT solution (0.5 mg/mL) to each well. The culture medium was removed after 4 h and 150 μL of DMSO was added to each well to dissolve the formazan crystals. Then the absorbance at 490 nm was measured with a microplate reader.

RESULTS AND DISCUSSION

Response of Probe 1 to Tyrosinase. Absorption and fluorescence spectra of probe 1 before and after adding tyrosinase were investigated. As shown in Figure 2A, probe 1 exhibits an absorption band at 475 nm, and after addition of tyrosinase a new absorption band at 570 nm appears concomitant with a color change from colorless to pink. As shown in Figure 2B, probe 1 shows almost no emission, due to the protection of 7-hydroxy in resorufin,[22,23] and fluorescence intensity at 583 nm enhances

significantly after addition of tyrosinase. As shown in Figure S1, resorufin exhibits an absorption band at 570 nm and an emission band at 583 nm, demonstrating that the response originates from the generation of resorufin. Kojic acid is an inhibitor of tyrosinase.[26] The presence of kojic acid leads to a weaker response (Figure S2A), and kojic acid has no effect on the fluorescence intensity of resorufin and probe 1 (Figure S2B), indicating that the response is ascribed to tyrosinase-catalyzed reaction of probe 1.

The experimental conditions were then optimized, including pH, temperature and time. As shown in Figure S3A, the change in pH, especially in pH 7–8, affects the response of probe 1 to tyrosinase seriously, and the optimal response is obtained at pH 8.0. As shown in Figure S3B, temperature also has some effect on the response, and the optimal response is achieved at temperature 37–42 □. Probe 1 is intended for application in cells, and thus physiological conditions (pH 7.4 and temperature 37 □) are used in this study. As shown in Figure S4A, time course reveals that tyrosinase-catalyzed reaction of probe 1 is time-dependent, and 5 h is employed in subsequent experiments with overall consideration of time and selectivity. Additionally, kinetic parameters for tyrosinase-catalyzed reaction of probe 1 were determined via Lineweaver-Burk analysis (Figure S4B). Michaelis constant (K_m) and maximum initial reaction rate (V_{max}) are calculated to be 6.5 μM and 0.9 nM min^{-1}, respectively.

Under the optimized conditions, the response of probe 1 to different concentrations of tyrosinase was investigated. As expected (Figure 3A), a gradual

increase of fluorescence intensity is observed with the increase of tyrosinase concentration. Besides, probe 1 exhibits a good linear response to tyrosinase in the range of 1–100 U mL^{-1} with detection limit of 0.5 U mL^{-1} (S/N = 3) (Figure 3B).

Response Mechanism. It has been reported that some boronic esters are prone to hydrolysis.[27,28] Therefore, the hydrolytic property of probe 1 was evaluated. As shown in Figure S5, after incubation at physiological conditions (pH 7.4 and temperature 37 ℃) for 5 h, the chromatographic peak intensity of probe 1 is not changed and no new chromatographic peak appears, indicating the hydrolytic stability of probe 1 under experimental conditions. This occurs because the hydrolysis of boronic esters is structure-dependent.[27,28]

The catalytic function of tyrosinase is hydroxylation at adjacent position, and the two copper ions in the active site of tyrosinase capture substrate via coordination.[3,4] Phenylboronic acid pinacol ester can coordinate with copper ions,[29] and thus we speculate that phenylboronic acid pinacol ester is a substrate of tyrosinase. As a result, phenylboronic acid pinacol ester in probe 1 is hydroxylated at adjacent position by tyrosinase followed by 1,6-rearrangement-elimination to produce resorufin and compound 2 (Figure 1).

To verify the above speculation, the reaction products of probe 1 with tyrosinase were analyzed with HPLC and HRMS. As shown in Figure S6, after addition of tyrosinase, the chromatographic peak of probe 1 decreases concomitant with the emergence of the chromatographic peak of resorufin. Additionally, HRMS of probe 1 after addition of tyrosinase displays the signals of resorufin ($M + H^+$, m/z 214.0496)

and compound 2 ($M + H^+$, *m*/*z* 233.1355) (Figure S7). These results demonstrate that the reaction of probe 1 with tyrosinase produces resorufin and compound 2, confirming the above speculation.

To further verify the above speculation, the reaction of phenylboronic acid pinacol ester with tyrosinase was investigated. As shown in Figure S8, after addition of tyrosinase, the chromatographic peak of phenylboronic acid pinacol ester decreases, confirming that phenylboronic acid pinacol ester is a substrate of tyrosinase, but the chromatographic peak of 2-hydroxyphenylboronic acid pinacol ester does not appear, because 2-hydroxyphenylboronic acid pinacol ester can further react with tyrosinase (Figure S9). 2-Hydroxyphenylboronic acid pinacol ester is not only a phenylboronic acid pinacol ester derivative but also a phenol derivative, and thus it can react with tyrosinase. These results further confirm the above speculation.

Selectivity. The response of probe 1 to other biological substances was investigated, including inorganic salts, glucose, vitamins, amino acids, creatine, urea, enzymes and ROS. As shown in Figure 4, it is observed that these biological substances including ROS have little effect on the fluorescence intensity of probe 1. The above result suggests the high selectivity of probe 1 toward tyrosinase, and this is attributed to that only tyrosinase has the function of hydroxylation.

Although H_2O_2 is not responded by probe 1, it has two-side influence on the response. On one hand, H_2O_2 can oxidize phenylboronic acid pinacol ester to phenol.[30,31] Phenol, the natural substrate of tyrosinase, consequentially can be hydroxylated at adjacent position by tyrosinase with a faster rate.[15] On the other hand,

excess H_2O_2 can further oxidize phenol to quinone.[17] As expected (Figure S10), after adding H_2O_2 (50 μM) the response increases, but with adding more H_2O_2 (100 μM) the response further becomes weaker. H_2O_2 and tyrosinase are mainly produced in different organelles[32,33], but they coexist in cells because of molecular diffusion. Thereby, H_2O_2 can affect the imaging of tyrosinase in cells.

Table S1 lists the comparison between probe 1 and reported fluorescent tyrosinase probes[9–16]. It is found that probe 1 exhibits high selectivity, and it is also one of the excellent probes for tyrosinase.

Fluorescence Imaging of Tyrosinase in Cells. To begin with, the cytotoxicity of probe 1 was evaluated using a conventional MTT assay. As shown in Figure S11, when B16 cells are treated with probe 1 at a concentration of no more than 2.5 μM for 24 h, no significant cytotoxic response (cell viability ≥ 85%) is observed. Thereby, 2.5 μM of probe 1 is used for cell imaging.

Then fluorescence imaging of tyrosinase was conducted in B16 cells, a melanoma cell line that expresses tyrosinase. As shown in the image a of Figure 5, B16 cells display almost no fluorescence, which benefits from the long wavelengths of probe 1. As shown in the images b and c of Figure 5, B16 cells treated with probe 1 exhibit strong fluorescence, but B16 cells treated with kojic acid and probe 1 show weak fluorescence, demonstrating that probe 1 can be used to monitor intracellular tyrosinase activity. Fluorescence imaging of tyrosinase in HepG2 cells (non-melanoma cell line) was also carried out. As shown in the image d of Figure 5, HepG2 cells treated with probe 1 exhibit almost no fluorescence, and this is because

the expression of tyrosinase is mainly limited to melanocytes.[7,8]

CONCLUSIONS

In summary, a fluorescent probe based on tyrosinase-catalyzed hydroxylation of phenylboronic acid pinacol ester is developed for the detection of tyrosinase in cells. The probe exhibits high selectivity for tyrosinase over other biological substances including ROS, but it does not avoid the interference of H_2O_2. This probe provides an alternative method for selective detection of tyrosinase in biosystem.

ACKNOWLEDGEMENTS

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ASSOCIATED CONTENT

Supporting Information. Additional information as noted in the text (Figures S1–S11, Table S1); ^{1}H NMR, ^{13}C NMR, HRMS of compound 1 and probe 1.

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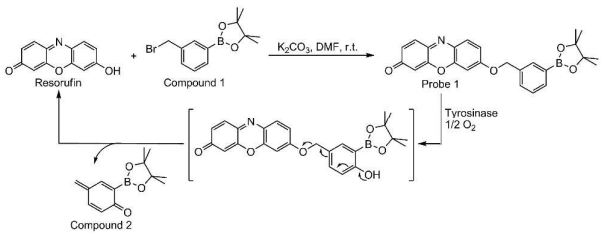

Figure 1. Synthetic route of probe 1and tyrosinase-catalyzed reaction of probe 1.

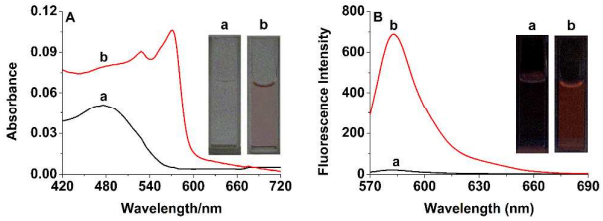

Figure 2. Absorption (A) and fluorescence (B) spectra of probe 1 (5 μM) before (a) and after (b) adding tyrosinase (150 U mL^{-1}) at 37 □ for 10 h in DMSO/PBS buffer (1/49 v/v, 10 mM, pH 8.0). λ_{ex} = 550 nm, slit widths = 3.0/3.0 nm.

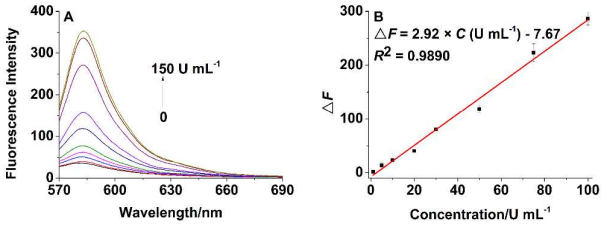

Figure 3. (A) Fluorescence spectra of probe 1 (5 μM) after adding different concentrations of tyrosinase (0, 1, 5, 10, 20, 30, 50, 75, 100, 150 U mL^{-1}). (B) Linear correlation between fluorescence enhancement and tyrosinase concentration (1–100 U mL^{-1}). $\lambda_{ex/em}$ = 550/583 nm, slit widths = 3.0/5.0 nm.

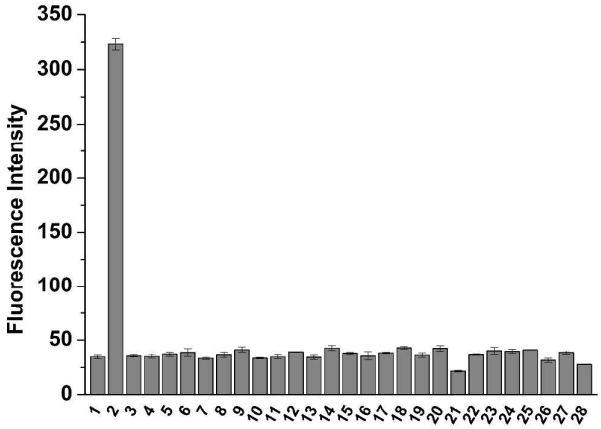

Figure 4. Fluorescence intensity of probe 1 (5 μM) after adding various substances. (1) none, (2) tyrosinase (150 U mL^{-1}), (3) KCl (150 mM), (4) $MgCl_2$ (2.5 mM), (5) $CaCl_2$ (2.5 mM), (6) $FeCl_3$ (100 μM), (7) glucose (10 mM), (8) vitamin C (1 mM), (9) vitamin B6 (1 mM), (10) glycine (1 mM), (11) glutamic acid (1 mM), (12) cysteine (100 μM), (13) glutathione (100 μM), (14) tyrosine (100 μM), (15) creatinine (10 mM), (16) urea (20 mM), (17) catalase (150 U mL^{-1}), (18) lipase (150 U mL^{-1}), (19) trypsin (15 mg L^{-1}), (20) glucose oxidase (150 U mL^{-1}), (21) H_2O_2 (50 μM), (22) TBHP (50 μM), (23) ClO^- (50 μM), (24) TBO· (50 μM), (25) HO· (50 μM), (26) $^1O_2$ (50 μM), (27) $O_2^{\cdot-}$ (50 μM), (28) $ONOO^-$ (50 μM). $\lambda_{ex/em}$ = 550/583 nm, slit widths = 3.0/5.0 nm.

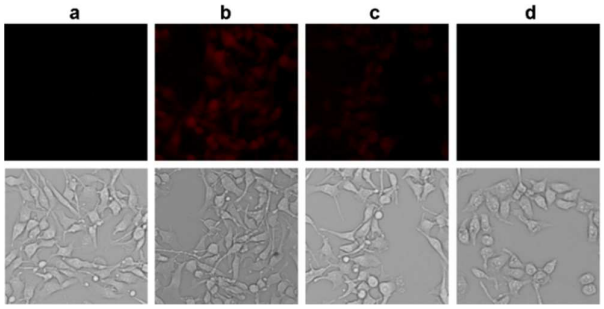

Figure 5. Fluorescence images. (a) B16 cells, (b) B16 cells treated with probe 1 (2.5 μM) for 5 h, (c) B16 cells pretreated with kojic acid (200 μM) for 2 h and then treated with probe 1 (2.5 μM) for 5 h, (d) HepG2 cells treated with probe 1 (2.5 μM) for 5 h. The second row shows the corresponding brightfield image.

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