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A far-red fluorescent probe for sensing laccase in fungi and its application in developing an effective biocatalyst for the biosynthesis of antituberculous dicoumarin†

Zhenhao Tian,^{‡,ab} Qingsong Yan,^{‡,a} Lei Feng,^{ib,‡,abc} Sa Deng,^a Chao Wang,^{ib,*ab}
Jingnan Cui,^b Chunjie Wang,^a Zhanjun Zhang,^d Tony D. James^{ib,*c} and Xiaochi Ma^{ib,*a}

A far-red fluorescent probe has been developed for sensing fungal laccase. The probe was used to determine that *Rhizopus oryzae* had a high level endogenous laccase amongst 24 fungal strains. The *Rhizopus oryzae* was then used as a biocatalyst for the preparation of dicoumarin resulting in significant inhibition of *Mycobacterium tuberculosis* H37Ra.

Laccase (EC 1.10.3.2) belongs to the family of copper-containing phenol oxidases with molecular masses ranging from 50 to 130 kDa.¹ As a well-known biocatalyst, laccase could catalyze the oxidation of various organic compounds, such as diphenols, polyphenols, diamines, aromatic amines, and benzenethiol derivatives during a four-electron transfer processes while reducing O₂ to water.² Laccase is generally found in higher plants, fungi, bacteria, and even insects.³ In particular, laccase activity has been observed in numerous fungal species with a majority of laccases having been isolated from around 60 fungal strains, such as deuteromycetes, ascomycetes, and basidiomycetes.³

Several phenolic substrates have been exploited for colorimetric assays of laccase, including *para*-cresol, diaminobenzidine, guaiacol, *paraphenylenediamine*, *para*-quinol, syringaldazine, and *ortho*-toluidine, based on the formation of highly colored products.⁴ However, colorimetric methods have some disadvantages such as interference with the substrates absorption, enzyme inactivation, and pH-dependent formation, all of which limit the accuracy and precision of these techniques.

Enzyme-activated fluorescent probes have numerous advantages, including superior sensitivity, wide linear dynamic range, rapidity, high accuracy, and selectivity, and thus have attracted considerable research interest and been used to sense endogenous enzymes in various complex bio-samples.⁵ Fluorescent probes with far-red wavelength are particularly attractive because of their inherent optical properties (excellent tissue penetration, low autofluorescence, and minimal light induced biological damage).⁶ As far as we know, only one fluorescent probe Amplex Red has been applied to the assay the laccase activity from soil with a fluorescence at around 583 nm.⁷ Therefore, the development of practical fluorescent probes with far-red wavelength emission for the detection of laccase especially in fungal laccase is highly desirable.

In the present work, we have developed a fluorescent probe (**DDAN**) by addition of *N*-acetyl group to 7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one) (**DDAO**), according to the structural feature and substrate preference of laccase. After the oxidation of **DDAN** by laccase, the formation of a ketone and dissociation of acetyl group result in the production of **DDAO**, a well-known far-red fluorophore suitable for imaging in living systems. **DDAN** can be used to detect laccase activity, and image laccase in fungal mycelia and fungal colonies in real-time. Furthermore, we fully demonstrate the feasibility and practicability of **DDAN** as a real-time assay to determine preliminary laccase activity in various fungi to discover particularly active enzymes. Using the fungal laccase as the biocatalyst, a dicoumarin was prepared successfully, which displayed potential inhibitory effects on *Mycobacterium tuberculosis* H37Ra.

Fungal laccase can oxidize non-fluorescent **DDAN** under a radical mechanism triggered by the phenolic hydroxyl group in the presence of oxygen. Then the intramolecular rearrangement of **DDAN** results in the loss of the *N*-acetyl group, which leads to the production of the highly fluorescent **DDAO** (Fig. 1a). The product, **DDAO** is violet under sunlight and red under a handheld UV lamp (365 nm), facilitating the potential detection of laccase using the naked-eye (Fig. 1b). The fluorescence spectrum of **DDAO** indicates a far-red fluorescence emission with maximum emission at 658 nm ($\Phi_{\text{DDAO}} = 0.40$) excited by

^a National-Local Joint Engineering Research Center for Drug-Research and Development of Neurodegenerative Disease, Academy of Integrative Medicine, College of Pharmacy, Dalian Medical University, Dalian, 116044, China.
E-mail: wach_edu@sina.com, maxc1978@163.com

^b State Key Laboratory of Fine Chemicals, Dalian University of Technology, Dalian 116024, China

^c Department of Chemistry, University of Bath, Bath BA2 7AY, UK.
E-mail: t.d.james@bath.ac.uk

^d State Key Laboratory of Cognitive Neuroscience and Learning, Beijing Normal University, Beijing, China

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‡ These authors contributed equally to this work.



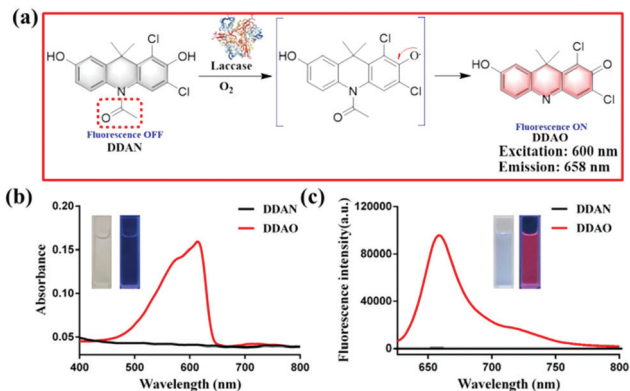


Fig. 1 (a) Proposed mechanism of the sensing of fungal laccase using **DDAN**. (b) Absorbance spectra of **DDAN** and **DDAO**. (c) Fluorescence spectra of **DDAN** and **DDAO** ($\lambda_{\text{ex}} = 600 \text{ nm}$).

laser at 600 nm (Fig. 1c).⁸ As shown in Fig. S7 (ESI[†]), both **DDAN** and **DDAO** were detected in the enzymatic system using HPLC, and no other products were observed. Thus, the fluorescent probe **DDAN** could be transformed by fungal laccase resulting in the production of the far-red fluorophore of **DDAO**.

The fluorescence spectra of **DDAN** towards increasing concentrations of fungal laccase (0–1.5 mg mL⁻¹) are shown in Fig. S8a (ESI[†]), and display a concentration dependent increase with laccase. Furthermore, on the basis of the fluorescence response of **DDAN** towards laccase at different incubation times, the time-dependent enzymatic reaction was confirmed as the oxidation of **DDAN** by laccase.

Interference by various species on the fluorescence intensity of **DDAN** and the enzymatic co-incubation of **DDAN** with laccase was then investigated. As shown in Fig. S9 (ESI[†]), various metal ions, chloroplatinate ions, several amino acids, together with EDTA (2 mM) displayed no influence on the fluorescence intensity of the bioassay system containing **DDAN** and laccase, all of which revealed that the fungal laccase resulted in the oxidation of **DDAN** in complex systems. In consideration of the enzymatic reaction mechanism of oxidation, some oxidants and reductants were investigated for their interference on the fluorescence intensity of **DDAN** and the bioassay system of laccase. Neither the oxidants (KMnO₄ and H₂O₂) or the common reductants (vitamin C, Na₂S₂O₃, DTT and GSH) influenced the fluorescence intensity of **DDAN** (Fig. S9c, ESI[†]). Finally, **DDAN** displayed specificity towards laccase in the presence of several other biological enzymes and proteins (Fig. S9d, ESI[†]). The kinetic profile for the oxidation of **DDAN** by laccase was determined to follow Michaelis–Menten kinetics, with K_m $72.56 \pm 9.323 \mu\text{M}$ and V_{max} $92166 \pm 3781 \mu\text{mol min}^{-1} \text{mg}^{-1}$, indicating a good affinity for **DDAN** towards fungal laccase (Fig. S10, ESI[†]). Clearly demonstrating that the far-red fluorescent probe **DDAN** can be used to assay laccase activity sensitively and selectively *in vitro*.

Encouraged by these results, 24 fungal strains were cultured to determine the endogenous fungal laccase as well as the laccase activity using **DDAN**. Compared with the control groups without **DDAN**, most of the 24 fungal mycelia displayed fluorescence with differing intensities (Fig. 2 and Fig. S13–S22, ESI[†]).

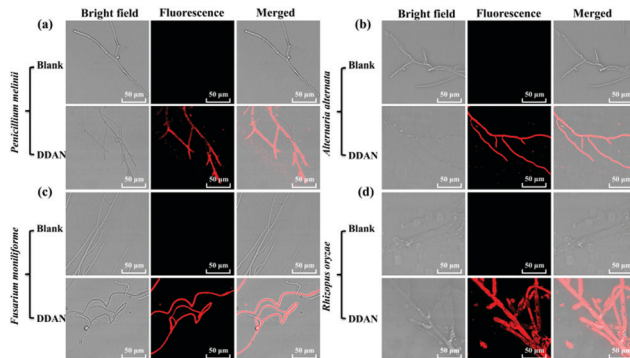


Fig. 2 Confocal laser scanning microscopy (CLSM) images of fungal mycelia in the presence of **DDAN** (50 μM). (a) *Penicillium melinii*; (b) *Alternaria alternata*; (c) *Fusarium moniliforme*; (d) *Rhizopus oryzae*. Excitation, 633 nm; emission, 645–690 nm. Scale bar: 50 μm .

For example, *Penicillium melinii*, *Alternaria alternata*, *Fusarium moniliforme*, and *Rhizopus oryzae*, all displayed strong red fluorescence signals, clearly indicating the production of **DDAO** due to the endogenous laccase activity. Additionally, the current fluorescent probe **DDAN** displayed no cytotoxicity towards fungal cells at 100 μM after co-incubation of 24 h (Fig. S23, ESI[†]).

Fungal colonies cultured on YPD agar plates are convenient for morphologic observation, fungal strain preservation, and the isolation and identification of target fungal strains. So the sensing of fungal laccase in plates could be helpful for identification and isolation of fungi expressed laccase from complex samples. Therefore, **DDAN** (50 μM) was incubated (30 °C, 2 h) with fungal colonies and fungal mycelia of four fungal strains on plates. As shown in Fig. 3, different fluorescence intensities were observed for these fungal colonies. Clearly demonstrating

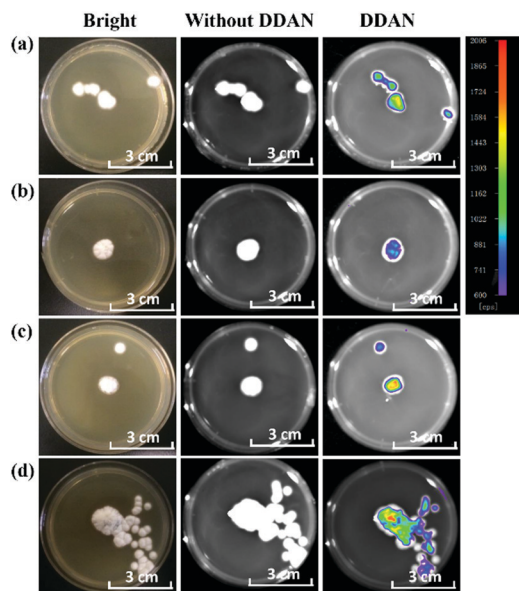


Fig. 3 Fluorescence images of fungal colonies in the presence of **DDAN** (50 μM). (a) *Penicillium melinii*; (b) *Alternaria alternata*; (c) *Fusarium moniliforme*; (d) *Rhizopus oryzae*. Excitation, 630 nm; emission, 665–735 nm.



produced by fungal laccase could potentially be developed into an anti-tuberculosis candidate.

In summary a far-red fluorescent probe **DDAN** was designed and developed to detect fungal laccase in various complex systems. **DDAN** could be used to measure the laccase activity, and selectively monitor the endogenous laccase of fungi in real time. The fluorescent probe **DDAN** was used to investigate laccase activity in 24 fungal strains. Among these fungi *Rhizopus oryzae* As 3.2028 was found to be a fungal strain with particularly high expression of laccase. We then used this over expressing laccase system as a biocatalyst for the production of dicoumarin from 6,7-dihydroxymarin. The catalytic mechanism was evaluated using kinetics and *in silico* docking analysis. It was found that dicoumarin could significantly inhibit *M. tuberculosis* H37Ra, where the GlmU protein was the potential active target, indicating that dicoumarin could be used as a potential anti-tuberculosis agent. Most notably, **DDAN** a far-red fluorescent probe has facilitated the evaluation of endogenous fungal laccase and allowed us to develop new drug candidates based on biotransformed bioactive products.

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Conflicts of interest

There are no conflicts to declare.

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