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An unnatural amino acid based fluorescent probe for phenylalanine ammonia lyase†

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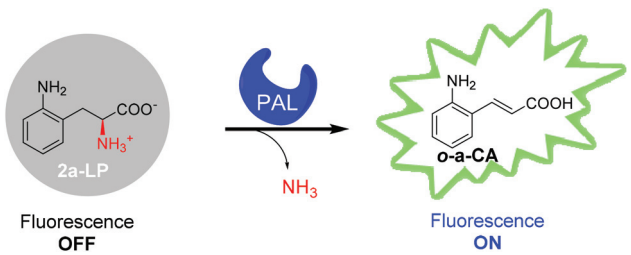
A fluorescent probe (2a-LP) based on an unnatural amino acid (UAA) is developed for the detection of phenylalanine ammonia lyase (PAL). In the presence of PAL, 2a-LP is catalytically deaminated to *ortho*-amino-transcinnamic acid (*o*-a-CA), which shows a remarkable "off–on" fluorescence signal. Thus, the probe 2a-LP enables direct visualization of the PAL activity in tomato under UV illumination and has potential *in vitro* assays.

Phenylalanine ammonia-lyase (PAL) is a non-hydrolytic enzyme and catalyses the biotransformation of L-phenylalanine (L-Phe) to *trans*-cinnamic acid (*t*-CA) and ammonia in the absence of exogenous cofactors.[1a] PAL is widely distributed in higher plants, fungi, yeasts and prokaryotes (*Streptomyces*), but absent in eukaryotic bacteria and animal tissues.[1b] PAL is employed in enzyme replacement therapy (ERT) for phenylketonuria (PKU).[2] PAL plays an important role in the catabolism of microorganisms, allowing them to use L-Phe as a sole source of carbon and nitrogen.[3] PAL is also involved in the biosynthesis of salic acid (SA) and phenylpropanoids, which play fundamental roles in plant immune response, development and protection against environmental stresses.[4a,b] Expression of PAL is rapidly induced during plant–pathogen interactions, and inhibition of PAL activity results in its breakdown.[4c] The determination of PAL activity is therefore of significance and can be used to monitor the plant induced resistance (IR).

PAL activity is typically estimated by the level of *t*-CA, which is measured by UV-Vis spectroscopy. However, coumarin based compounds have similar absorption maximum to *t*-CA and constitute strong basal interferences.[5] High performance liquid chromatography (HPLC) is a robust alternative for *t*-CA measurement. Another approach is to examine PAL gene expression. One usually uses specific reporter molecules, which are induced upon the activation of the PAL promoter, to analyse the PAL activity.[6a] For example, β-glucuronidase (GUS) or luciferase reporter enzymes have been used to examine PAL gene expression in transgenic tobacco, bean and *A. thaliana*.[6b] Rookes reported a GFP probe for PAL.[6c] Compared to conventional HPLC assays and GFP probes, small-molecular fluorescent probes are widely used in detecting, tracing, and visualizing the function of enzymes because of their insignificant steric bulk, fast labelling kinetics and characteristics such as real-time determination, high selectivity and sensitivity.[7] Thus, developing specific fluorescent probes for PAL is of considerable interest.

Deamination of L-Phe by PAL results in efficient conjugation between the carboxyl and the benzene ring. We envisage that incorporation of an electron donating group at the *ortho*/*para* position of the benzene ring will build an electronic push–pull system, which is typically a good dye and hopefully a good fluorophore. Based on the aforementioned design rationale, we synthesized an L-Phe derivative, 2-amino-L-phenylalanine (**2a-LP**), as a PAL probe (Scheme 1).

As shown in Scheme 1, upon deamination by PAL, **2a-LP** would release an *ortho*-amino-transcinnamic acid (*o*-a-CA), which emits blue fluorescence upon UV excitation. Utilizing this unnatural amino acid (UAA) as a PAL substrate, the PAL activity can be monitored. This is the first report using a synthetic UAA as a fluorescent probe for the direct detection of PAL activity. Previously, UAAs with unique small bioorthogonal handles have been frequently used in site-specific labelling of biomacromolecules,[8] and this new UAA based enzyme probe broadens the scope of applications of UAAs.



Scheme 1 Proposed mechanism of **2a-LP** as PAL probe.

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2a-LP and *o*-a-CA were synthesized as shown in Schemes S1–S2 (ESI†), and their structures were confirmed from ^{1}H NMR, ^{13}C NMR, and HRMS spectra (Sections S2 and S3, ESI†).

The absorption and fluorescence spectra of the probe and its enzymatic products were measured. *o*-a-CA emitted fluorescence at 494 nm with excitation at 335 nm and showed a 159 nm Stokes shift, and had similar fluorescence properties to *trans*-2-amino-5-hydroxymethyl-cinnamic acid.[9] The pH titration of *o*-a-CA showed that its fluorescence is stable between pH values of 6 and 10. The fluorescence quantum yield and the pK_a of *o*-a-CA were calculated to be 0.106 and 4.7 in water, respectively (Table S1, Fig. S2, ESI†). Since **2a-LP** is non-fluorescent, a significant "off–on" signal is expected if **2a-LP** can be catalysed and deaminated by PAL.

Then the probe, **2a-LP** was tested to detect the PAL activity in buffer solution (Tris-HCl, 50 mM). As the commercial PAL (*Rhodotorula glutinis* source) works at an optimal pH of 8.5, 0.5 mM Tris-HCl (pH 8.5) buffer solution was used in the subsequent experiments. **2a-LP** was incubated with 0.132 U mL^{-1} PAL solution for 10 h at 30 °C and the fluorescence intensity was measured. The fluorescence intensity of the above solution showed 13-fold enhancement after 2 h, and can be visualized by the naked-eyes under illumination with an UV lamp. In order to estimate the conversion of **2a-LP**, we run the assay and plot fluorescence intensity *versus* concentration of *o*-a-CA for each concentration (Fig. S3, ESI†). It was showed that 35.2% of **2a-LP** had been deaminated after 2 h, and 98.0% of **2a-LP** had been consumed after 10 h. The results demonstrated that **2a-LP** could be converted by PAL efficiently (Fig. 1).

Furthermore, the enzymatic metabolite of probe, **2a-LP** was analyzed by HPLC (Fig. 2). As expected, the retention peak of probe's enzymatic product is consistent with that of *o*-a-CA, and the retention time of *o*-a-CA and enzymatic product of **2a-LP** is 10.08 min and 10.05 min, respectively. The results further confirmed the proposed mechanism of **2a-LP** as a PAL probe.

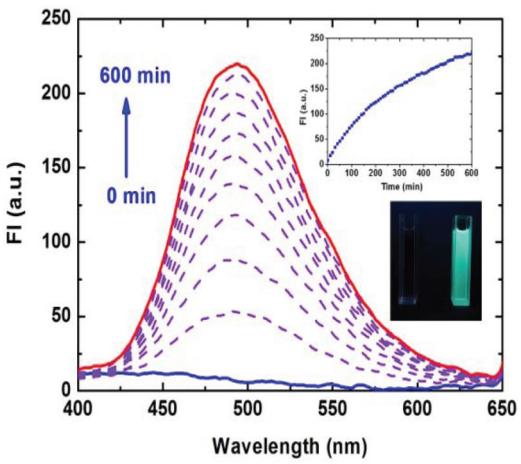

Fig. 1 Time course of fluorescence spectra of probe in the presence of PAL. Conditions: **2a-LP** (5 μM) with PAL (0.132 U mL) in 50 mM Tris-HCl buffer (pH 8.5), λ_{ex} = 335 nm, slit: 10/10 nm. Spectra were measured automatically every 10 min.

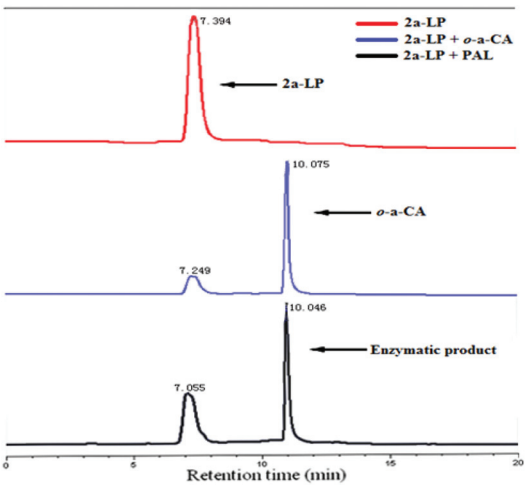

Fig. 2 Reversed-phase HPLC analysis of the PAL catalyzed deamination reaction of **2a-LP**. Conditions: 50 mM Tris-HCl buffer (pH 8.5), detected by UV 235 nm. (Red line) 0.5 mM **2a-LP** in Tris-HCl buffer; (blue line) 0.5 mM **2a-LP** and 0.5 mM *o*-a-CA in Tris-HCl buffer; (black line) 1 mM **2a-LP** incubated with PAL in Tris-HCl buffer for 5 h.

Next, we investigated whether the progress of the enzymatic reaction of **2a-LP** could be kinetically monitored by fluorometry. The fluorescence spectra of the **2a-LP** were periodically recorded during incubation of **2a-LP** with PAL, and the consumption of **2a-LP** by PAL led to a progressive increase in fluorescence intensity which could be followed in a continuous manner (Fig. S4, ESI†). Behaviours of **2a-LP** towards PAL show that fluorescence intensity is proportional to the enzyme activity within two hours' incubation (Fig. S5, ESI†). To establish a method for rapid detection of PAL activity, fluorescence intensity after one hour's incubation was chosen to establish a calibration curve (Fig. 3a). Combining the calibration curve and the fluorescence spectrum of an unknown sample incubated with **2a-LP**, the PAL activity could be estimated quickly.

With PAL, the enzymological properties of **2a-LP** have been studied using a continuous assay. After confirming the linearity of the integrated fluorescence response caused by various concentrations of *o*-a-CA (Fig. S3, ESI†), it was found that the initial reaction velocities were proportional to the PAL concentration (Fig. S6, ESI†). Then a suitable enzyme/substrate ratio was established, and the substrate concentration was varied to generate a saturation curve for determining the K_m value of **2a-LP**. The alternation of the initial velocity with the concentration of **2a-LP** was demonstrated by the Michaelis–Menten equation and the Lineweaver–Burk plot, and the result was calculated as K_m of 1.570 mM and V_{max} of 0.245 μM min^{-1} (Fig. 3b). This K_m value of **2a-LP** is 2.4-fold of its natural substrate, and the *para*-substituted L-Phe has smaller K_m values (Table S2, ESI†). This might be due to the fact that *ortho*-amino in **2a-LP** interfered with binding with a 4-methylidene-imidazole-5-one group (MIO) cofactor of PAL, and the better affinities of *para*-substitutes also provide us with further study orientation.

Next, **2a-LP** was used to monitor the activity of PAL in plant samples. Ultraviolet A (UV-A) induced plant samples and blank

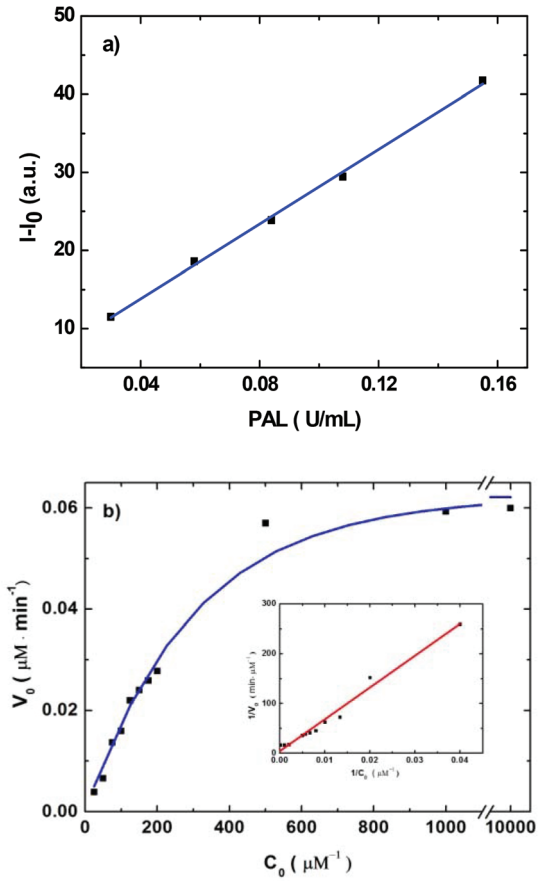

Fig. 3 (a) Fluorescence calibration curve for PAL activity. Probe (5 μM), PAL (0.026 U mL^{-1}, 0.050 U mL^{-1}, 0.071 U mL^{-1}, 0.092 U mL^{-1}, 0.132 U mL^{-1}), the estimated linear equation is $y = 207.6x + 8.5$ (r^2= 0.994). (b) Fluorimetric assay of PAL and **2a-LP**: Michaelis–Menten and Lineweaver–Burk plot (insert). PAL (0.012 U mL^{-1}), **2a-LP** (20–10 000 μM). Initial velocity values were calculated from the reaction time courses and based on the respective fluorescence signal. Initial velocity values were fitted to the Michaelis–Menten plot and to the Lineweaver–Burk plot, estimated linear equation $y = 6402.638x + 4.0778$, $r^2 = 0.979$). Other conditions: Tris-HCl buffer (50 mM, pH 8.5), 30 °C, λ_{ex} = 335 nm, λ_{em} = 494 nm, slit: 10/10 nm.

plant samples were designed because ultraviolet UV-A is an environmental stimulus, which can increase the expression of PAL gene in tomato.[10] **2a-LP** was applied in detection of the PAL activity in the UV-A irradiated tomato samples (Section S2, ESI†). **2a-LP** (5 μM) was incubated with UV-A irradiated and non-irradiated samples, and it was observed that the fluorescence intensity of UV-A irradiated samples showed 3.3-fold enhancement than that of non-treated samples (Fig. S7, ESI†). Combining the increased intensity after 1 hour with the calibration curve, we estimated the PAL activity of the UV-A irradiated sample as 0.035 U mL^{-1}, and the non-UV treated sample showed no difference with the negative control (Fig. 4). Also the UV-A irradiated sample mixed with L-Phe (5 μM) has been tested and it showed no obvious fluorescence intensity change (Fig. 4). This result might be due to the interference of L-Phe. L-Phe acts as a competitive inhibitor hindered **2a-LP** binding with PAL, it also confirmed that the fluorescence

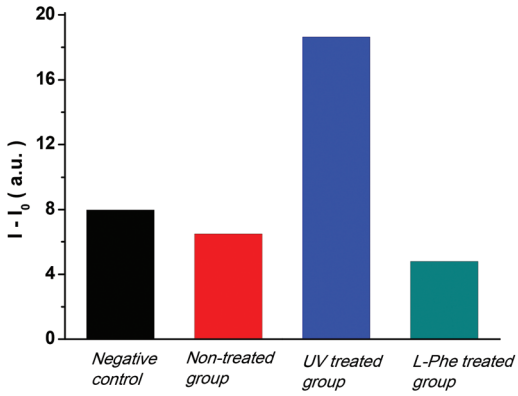

Fig. 4 Fluorescence responses of probe with tomato samples. Conditions: incubated **2a-LP** (5 μM) with samples for 1 h at 30 °C, λ_{ex} = 335 nm, λ_{em} = 494 nm, slit = 10/10 nm. Black column: the PAL extraction was from non-treated tomatoes without addition of probe, red column: incubated **2a-LP** with the PAL extraction from none UV treated tomatoes, blue column: incubated **2a-LP** with the PAL extraction from UV treated tomatoes, dark green column: incubated **2a-LP** with the PAL extraction from UV treated tomato with addition of L-Phe (5 μM).

enhancement in the UV irradiated sample was caused by the catalytic activity of PAL. This result demonstrates that **2a-LP** has great potential in the detection of PAL activities in plant samples, which is of significance in plant induced resistance research.

PAL has also been an important ERT tool for PKU,[2c] monitoring PAL activity under physiological conditions is of great significance for PKU ERT research. Therefore, we also explored the potential applications of **2a-LP** in biological systems. Incubation of HeLa cells with PAL for 3 hours at 37 °C was followed by the addition of **2a-LP** or none, and then incubation for another 30 min. As shown in Fig. 5, HeLa cells with only PAL

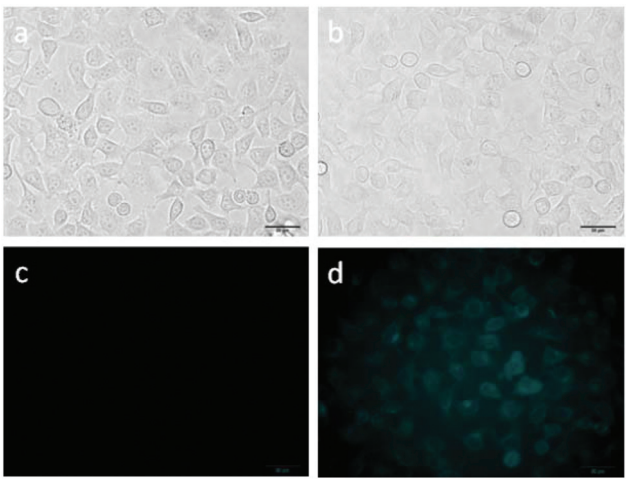

Fig. 5 Image of PAL detection in HeLa cells using **2a-LP** at 37 °C. (a) Bright-field image of HeLa cells incubated with PAL for 3 h. (b) Bright-field image of HeLa cells incubated with PAL for 3 h with **2a-LP** (100 mM) added for the final 30 min. (c) Fluorescence image of HeLa cells incubated with PAL for 3 h. (d) Fluorescence image of HeLa cells incubated with PAL for 3 h with **2a-LP** (100 mM) added for the final 30 min.

showed low fluorescence, while in the presence of PAL and **2a-LP**, HeLa cells show strong fluorescence, which also suggest that PAL can be monitored intracellularly by **2a-LP**. This result demonstrates that **2a-LP** has potential in visualizing PAL level changes of living cells, and **2a-LP** can be a novel tool for PKU ERT research.

Conclusions

In summary, we have developed a new fluorescent probe, **2a-LP**, based on the modification of PAL natural substrate, L-Phe, for PAL activity. Enzymatic deamination can be monitored upon mixing the probe and samples, without any additional treatment. Successful monitoring of this enzymatic activity is realized by utilizing the UAA deamination mechanism for the first time. This probe can be applied to monitor PAL activity in both plant samples and HeLa cells, and perhaps be a promising tool for the detection of PAL activity and plant induced resistance research.

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