Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

Unnatural Amino Acid Based Fluorescent Probe for Phenylalanine Ammonia Lyase

Zhenlin Tian, Weiping Zhu,* Yufang Xu,* and Xuhong Qian

State Key Laboratory of Bioreactor Engineering, Shanghai Key Laboratory of Chemical Biology, School of Pharmacy,
East China University of Science and Technology, Shanghai 200237, China.
Fax: +86 21 6425 2603; Tel: +86 21 6425 3822; E-mail: wpzhu@ecust.edu.cn.

Contents:

1. General Experimental Method

Materials and methods: All chemical regents and solvents were purchased from J&K, Alfa Aesar Corporation without other purification. 4-Amino-L-phenylalanine and L-2-Nitrophenylanine were purchased from MERYER Corporation, and purified with preparative HPLC-purification method 1. Phenylalanine Ammonia-Lyase (from Rhodotorula glutinis) was purchased from Sigma-Aldrich Corporation. Thin-layer chromatography (TCL) was performed on silica gel plates. Column chromatography was performed using silica gel (Hailang, Qingdao) 200-300 mesh. Stock solution of 2a-LP was prepared in Tris-HCl buffer (50 mM, pH 8.5). All the curves, lines were fitted by Origin, and the absorbance and fluorescence spectrum were smoothed by Origin.

Instruments: Fluorescence spectra were determined using a Varian Cary Eclipse fluorescence spectrometer. Absorption spectra were determined by a Varian Cary 100 UV-vis spectrophotometer. All pH measurements were made with a Sartorius basic pH-Meter PB-20. ^{1}H NMR and ^{13}C NMR spectra were recorded employing a Bruker AV-400 spectrometer with chemical shifts expressed in parts per million (in $CDCl_3$, CD_3OD, DMSO-d_6 and D_2O, TMS as internal standard). Electrospay ionization (ESI) mass spectrometry was performed in a HP 1100 LC-MS spectrometer. Analytical HPLC was performed on a ZoRBAX XDB-C18 column (Analytical 4.6×250mm 5-micrm, Agilent) with a HP 1100 system. Preparative HPLC was performed on with a XRs 10 C18 column (250×21.1 mm, Varian Pursuit) with a Galaxie chromatography data system.

Chromatography conditions

Preparative HPLC- purification method 1:

Sample (injection volume: 8 mL) was analyzed using a mobile phase composed of water(solvent A) and methanol(solvent B), according to the following gradient step(flow rate: 13 mL / min，detected by UV 235 nm)

0 min 90% A- 10% B

20 min 90% A- 10% B

25 min 0% A- 100% B

30 min 0% A- 100% B

Preparative HPLC- purification method 2:

Sample (injection volume: 8 mL) was analyzed using a mobile phase composed of water(solvent A) and methanol(solvent B), according to the following gradient step(flow rate: 13 mL/min，detected by UV 235 nm)

0 min 98% A- 2% B

20 min 98% A- 2% B

25 min 0% A- 100%B

30 min 0% A- 100%B

Analytical chromatography (HPLC):

Sample (injection volume: 10 μL) was analyzed using a mobile phase composed of 0.01 M ammonium acetate buffer at pH 6.0(solvent A) and acetonitrile(solvent B), according to the following gradient step(flow rate: 1.0 mL / min，detected by UV 235 nm)

0 min 100% A- 0% B

5 min 100% A- 0% B

20 min 0% A- 100%B

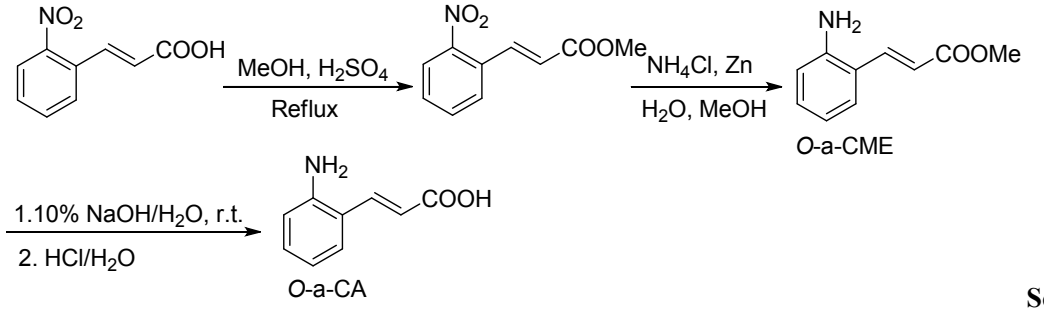
Extraction of PAL from tomatoes

Samples of tomatoes were bought from supermarket.12 tomato fruits were divided into two groups, none UV-A irradiated group (control group) and UV-A irradiated group (experiment group), and each group has six fruits. Their surfaces were cleaned with distilled water. The fruits of experiment group were irradiated by UV-A with two 16-watt UV lamps for 8 hours. Then all the fruits were kept in black plastic bags at room temperature for 2 days. The extraction of PAL from tomatoes was conducted according to the literature procedure[1]. The skin from three tomatoes (usually 2–5 g) was ground to a fine powder in a mortar and pestle using liquid N_2. The powder was then extracted with 20 mL 50 mM borate buffer (H_3BO_3 / $Na_2B_4O_7$) pH 8.8 containing 5% PVP (M_W 44,000), 18 mM mercaptoethanol. The homogenate was filtered and centrifuged at 10 000 r / min for 20 min.

References for this section:

[1] (a) J. Koukol and E. E. Conn, *J. Biol. Chem.*, 1961, **236**, 2692-2698. (b) C. E. Lister, J. E. Lancaster and J. R. L. Walker, *J. Am. Soc. Hortic. Sci.*, 1996, **121**, 281-285.

Preparation of *o*-a-CA and 2a-LP



Sc

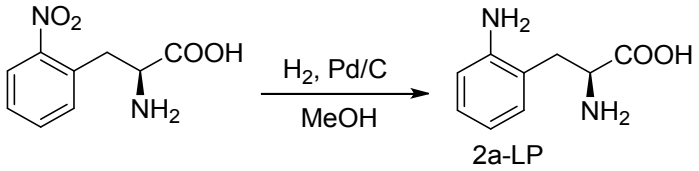
heme S1. Synthetic route of *O*-a-CA

Synthetic route of *O*-a-CA

(E)-methyl 3-(2-nitrophenyl)acrylate was prepared according to the literature procedure[2a]. In a 50 mL flask equipped with a reflux condenser, 1.931 g (1 mmol) of (E)-3-(2-nitrophenyl) acrylic acid and 20 mL methanol were mixed, 20 μL of 98% H_2SO_4 was then added dropwise. The mixture was refluxed for 6 h, and then cooled at room temperature. $NaHCO_3$ solution (0.5g dissolved in 2 mL water) was added to the above solution. Methanol was removed by using a rotary-evaporator and the residue was extracted with ethyl acetate, then dried with Na_2SO_4. After the removal of ethyl acetate, the white solid was collected. Yield 90%, M.P. 73 – 74 ^{0}C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.09 (d, J = 7.6 Hz, 1H), 7.95 (d, J = 16.0 Hz, 1H), 7.93 (d, J = 7.6 Hz, 1H), 7.79 (t, J = 7.6 Hz, 1H), 7.69 (t, J = 7.6 Hz, 1H), 6.65 (d, J = 16.0 Hz, 1H), 3.76 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 52.3, 122.7, 25.2, 129.7, 129.8, 131.5, 134.4, 140.1, 148.7, 166.4.

(E)-methyl 3-(2-aminophenyl)acrylate was prepared according to the literature procedure[1]. 0.90 g (4.34 mmol) of (E)-methyl 3-(2-nitrophenyl)acrylate, 20 mL of methanol, 2 mL of water, and 0.40 g of NH_4Cl were mixed in a 50 mL flask equipped with a reflux condenser. The mixture was heated and 2.79 g of Zinc powder was added slowly under stirring. The mixture was refluxed at 75–80 ^{0}C for 2 h, and finally the solvent was removed using a rotary-evaporator. The crude product was purified with Chromatogram Column (PE/EA=6:1),Yield 78%. M.P.65–66 ^{0}C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.90 (d, J = 15.6 Hz, 1H), 7.45 (d, J = 7.6 Hz, 1H), 7.08 (t, J = 7.6 Hz, 1H), 6.70 (d, J = 7.6 Hz, 1H), 6.54 (t, J = 7.6 Hz, 1H), 6.37 (d, J = 15.6 Hz, 1H), 5.62 (s, 2H), 3.71 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 167.6, 148.8, 141.3, 131.9, 127.8, 117.9, 116.9, 116.8, 115.5, 51.7.

trans-*O*-amino-cinnamic acid was prepared according to the literature procedure[1]. 0.20 g (1.13 mmol) of methyl b-(2-aminophenyl)propenoate and NaOH aqueous solution (5 g dissolved in 5 mL water) were mixed in a 10 mL round-bottom flask and stirred under the room temperature overnight . The pH of the solution was adjusted to 7 with dilute HCl aqueous solution, and the solvent was removed using a rotary-evaporator. The yellow solid was dissolved in 5 mL of Methanol. The filtrate was purified by HPLC, Yield 85%. M.P. 152–153 ^{0}C (decomposed). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.18 (s, 1H), 7.81 (d, J = 15.6 Hz, 1H), 7.42 (d, J = 7.6 Hz, 1H), 7.07 (t, J = 7.6 Hz, 1H), 6.69 (d, J = 7.6 Hz, 1H), 6.54 (t, J = 7.6 Hz, 1H), 6.27 (d, J = 15.6 Hz, 1H), 5.55 (s, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ: 171.1, 148.6, 142.4, 132.4, 128.5, 120.7, 119.2, 118.1, 117.8.



Scheme S2. Synthetic route of *O*-Aminophenyl-L-alanine

Synthesis of *2*-Aminophenyl-L-alanine (2a-LP)

***2*-Aminophenyl-L-alanine** was prepared according to the literature procedure[2]. In a 50 mL flask, 200 mg of 2-Amino-L-phenylalanine was dissolved in 30 mL of Methanol under the H_2 atmosphere of and catalyzed by Pd/C (5mg). The mixture was stirred at 20 ^{0}C for 6 h and filtered. Then the solvent was removed using a rotary-evaporator, and the crude product was purified by the HPLC-purification method 2. Yield 52%. M.P. 165-166 ^{0}C (decomposed), $[\alpha]_D^{21}$ -54.5^0(c = 0.5, 50% MeOH).M.P. 161-163 ℃。^{1}H NMR (400 MHz, D_2O) δ: 7.18 (t, J = 7.6 Hz, 1H), 7.12 (d, J = 7.6 Hz, 1H), 6.83-6.88 (m, 2H), 3.93-3.97 (m, 1H), 3.21-3.26 (m, 1H), 2.94-3.00 (m, 1H). 13C NMR (100 MHz, D_2O) δ: 174.6, 144.6, 131.1, 128.9, 121.2, 120.0, 117.6, 54.2, 32.4. HRMS (EI+) calcd for $C_9H_{12}N_2O_2$ $[M]^+$ 180.0899, found 180.0897.

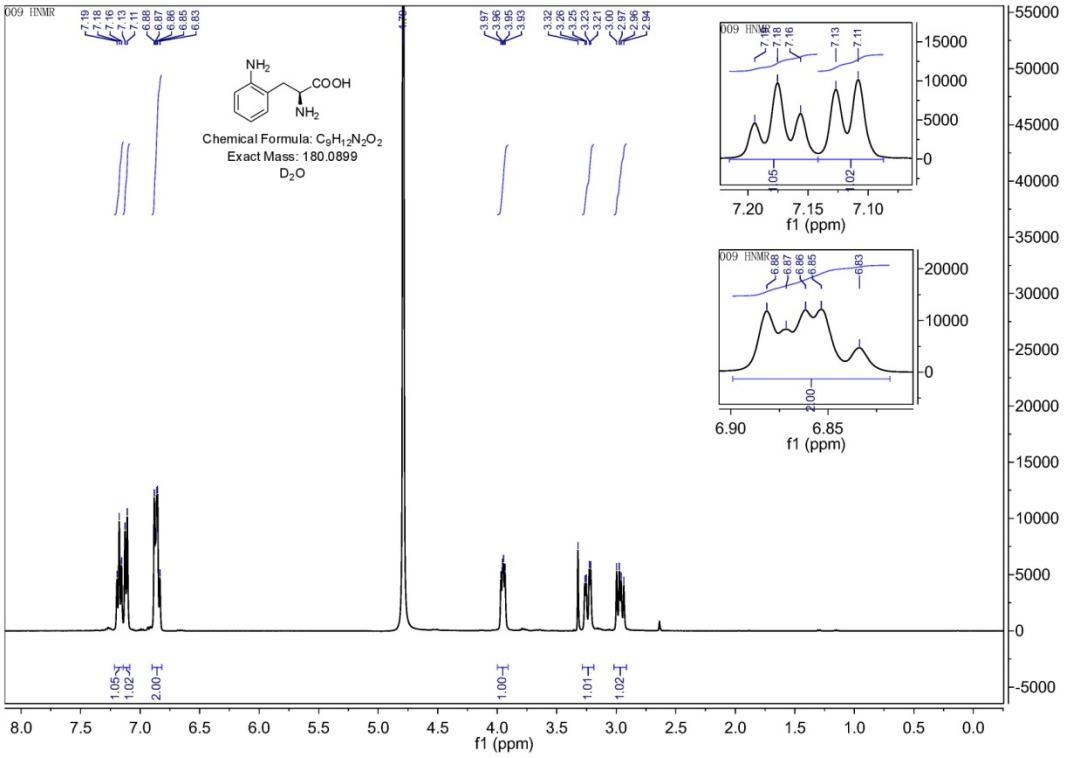
References for this section:

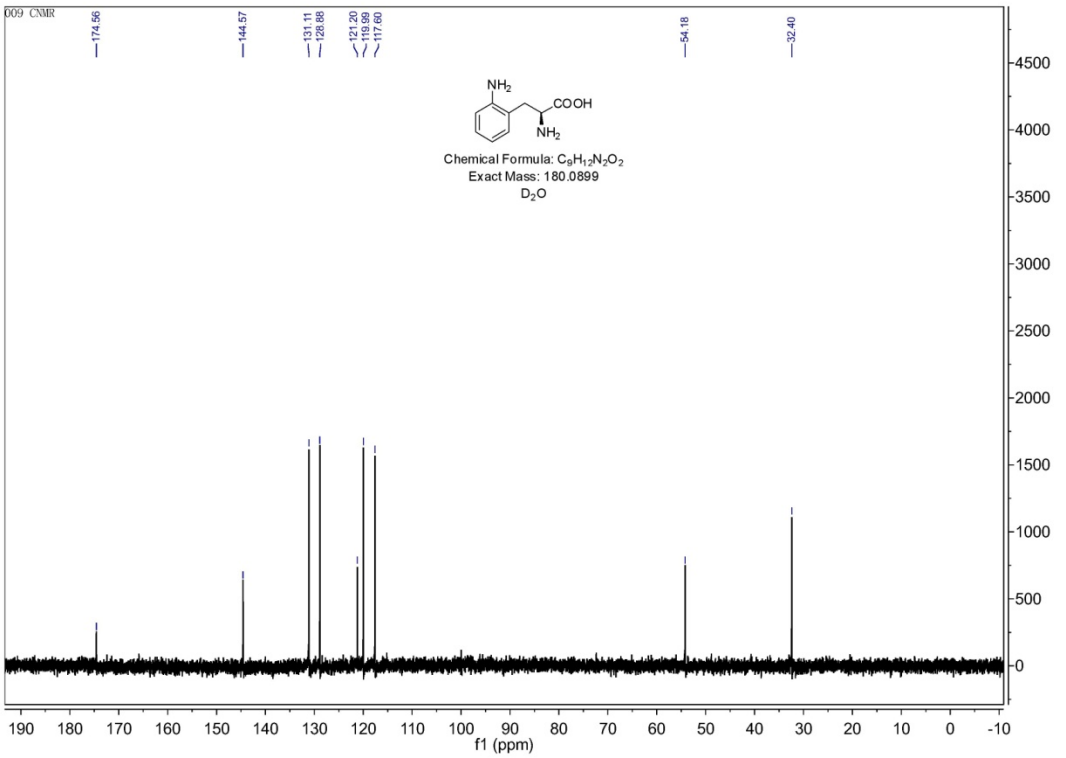
[1] (a) J. Koukol and E. E. Conn, *J. Biol. Chem.*, 1961, 236, 2692-2698. (b) D. N. Kuhn, J. Chappell, A. Boudet and K. Hahlbrock, *Proc. Natl. Acad. Sci. USA*, 1984, **81**, 1102-1106. (c) J. Guo and M. H. Wang, *Plant Growth Regul.*, 2010, **62**, 1-8.

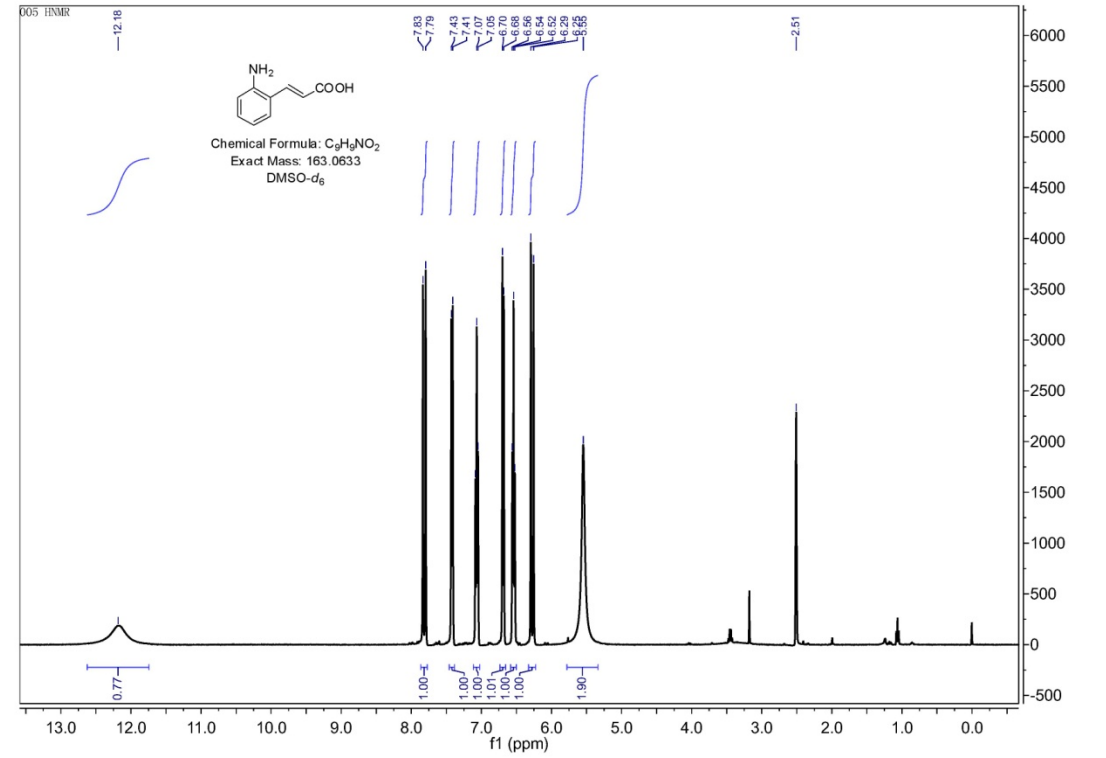
[2] (a) C. O. Saacutenchez, F. R. Diacuteaz, N. Gatica, C. Bustos, K. Espintildeeira and D. Huaquimilla, *Polym. Bull.*, 2011, **67**, 29-4343. (b) A. L. Davis, D. R. Smith,

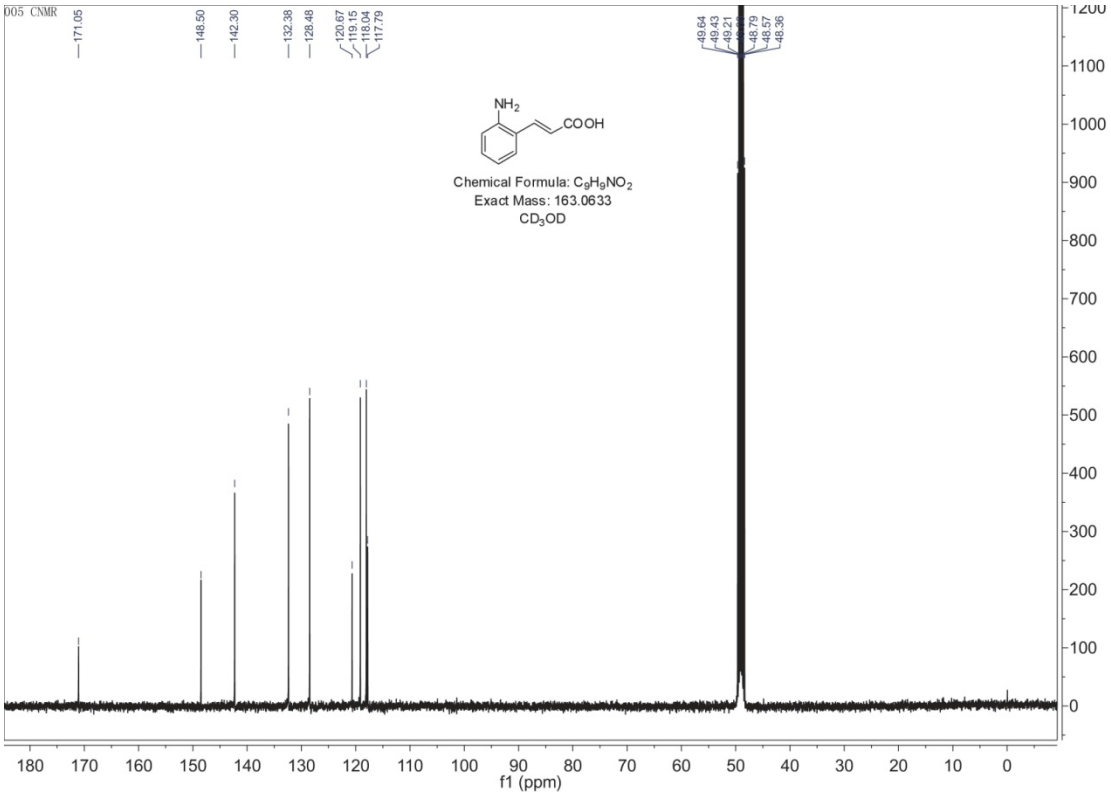
D. C. Foyt, J. L. Black and T. J. McCord, *J. Med. Chem.*, 1972, **15**, 325-327.

2. ^{1}H NMR and ^{13}C NMR of probe 2a-LP and its metabolic product









3. Protocols for enzyme assay

Reversed-phase HPLC analysis of the PAL catalyzed deamination

Stock solution of 2a-LP was prepared in Tris-HCl buffer (50 mM, pH 8.5). The reaction was started by addition of an appropriate volume of 2a-LP stock (5 μM) to the enzyme solution (0.014 U/mL). The solution was incubated at 30ºC for 5h. Samples were then analyzed by HPLC (Analytical method as described; retention times: 20 mins).

Protocol for fluorescence assays of enzyme activities

Stock solution of 2a-LP was prepared in Tris-HCl buffer (50 mM, pH 8.5). Appropriate concentrations enzyme solution was diluted from stock solution(280 μL, 2 uint in 60% glycerol, 3 mM Tris-HCl, pH 7.5, containing up to 0.5 M $(NH_4)_2SO_4$). In each experiment, the flouorescence intensity changes were recorded continiously. The data collection program was then re-started. And after 10 min, the enzyme reaction was initiated by addition of enzyme stock solution. Increased fluorescence values were obtained by the fluorescence deduced the beginning value. And the reaction velocities ware determined by the conversion velocities of 2a-LP, which were resulted from the increased fluorescence area peak. And the initial velocity is calculated by the average velocity between the first hour.

4. Additional tables, figures and schemes

Table S1 Quantum yields of related compounds

	PAL-01	PAL-02	PAL-03
Water (0.05% Methanol)	0.106	0.004	ND
Methanol	0.486	—	0.117

Fluorescence quantum yield standard: Quinine sulphate, 0.1 M H_2SO_4 in water, $\Phi = 0.546$, λ_{ex}= 366 nm.[a] [a]from J. Olmsted, *J. Phys. Chem.*, 1979, **83**, 2581-2584.

Table S2 Kinetic properties of phenylalanine ammonia lyase (*Rhodotorula glutinis source* **) for different substrate**

Substrate	K_m (mM)
L-Phenylalanine	0.65[b]
Tyrosine	0.20[b]
4-Fluoro-L-Phenylalanine	0.80[b]
4-Choro-L- Phenylalanine	0.76[b]

3-(2-Thienyl)-alanine	1.45[b]
2-amino-L-Phenylalanine	1.57

[b]from K. R. Hanson and E. A. Havir, *Arch. Biochem. Biophys.*, 1977, **180**, 102-113.

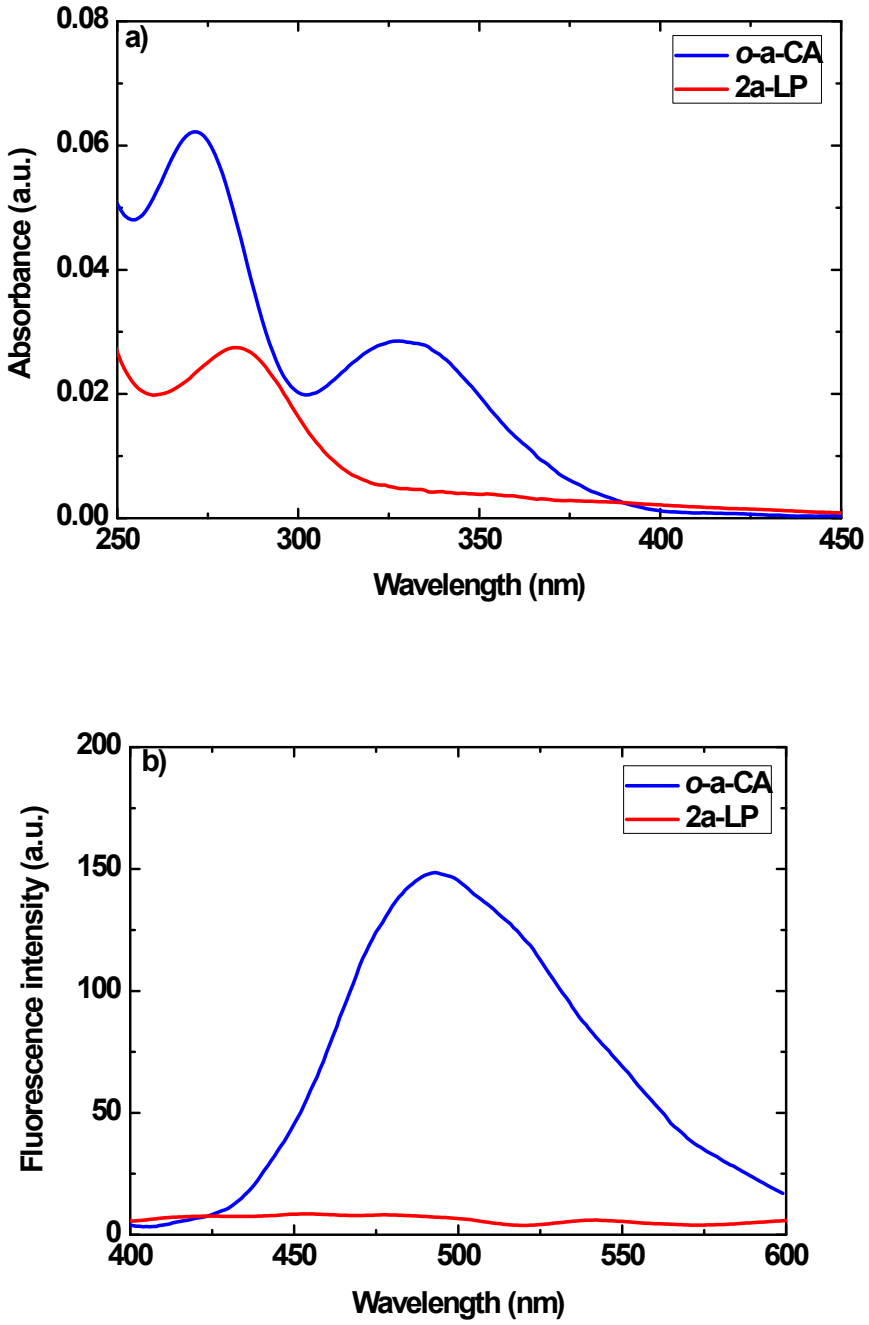


Fig. S1 (a) Absorbance spectra of 2a-LP (5 μM) and *O*-a-CA (5 μM) in Tris-HCl buffer (50 mM, pH 8.5). (b) Fluorescence spectra of 2a-LP (5 μM) and *O*-a-CA (5 μM) in 50 mM Tris-HCl buffer (pH 8.5), 30 ℃, λ_{ex}= 335 nm, slit: 5/5 nm.

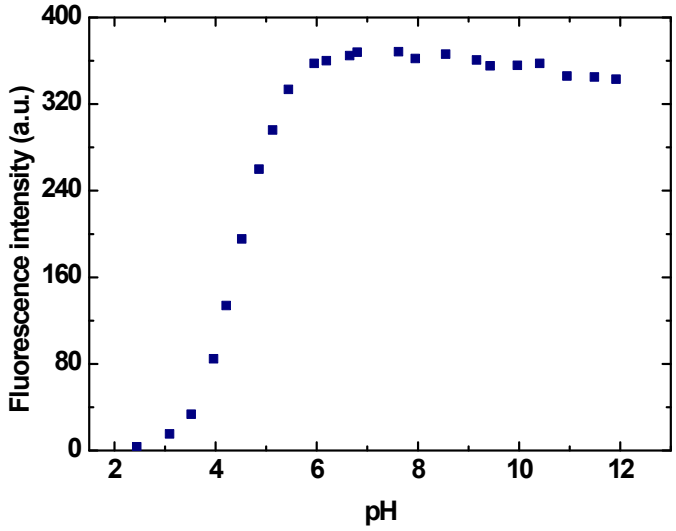


Fig. S2 Fluorescence response of *O*-a-CA (10 μM) to various pH in water. pH 2~12, 25 ℃, λ_{ex}= 335 nm, λ_{em}= 494 nm, slit: 5/5 nm.

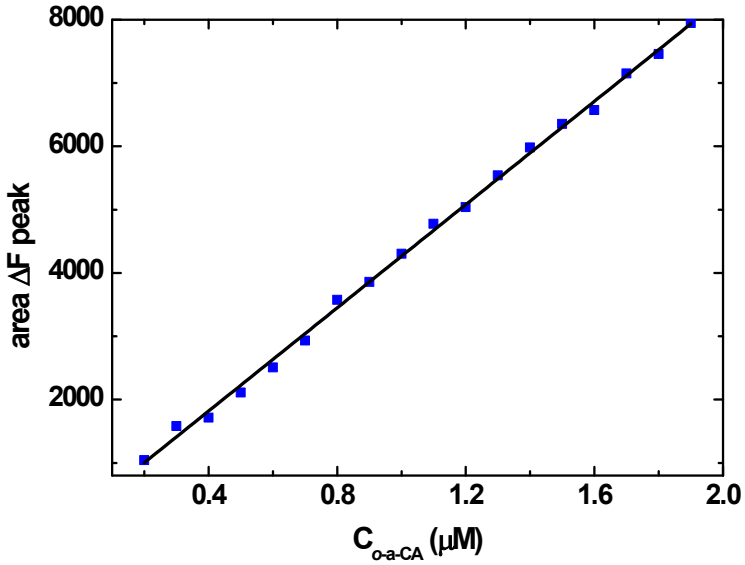


Fig. S3 Fluorescence assay of *o*-a-CA. Conditions: *o*-a-CA (0.2 μM, 0.3 μM, 0.4 μM , 0.5 μM, 0.6 μM, 0.7 μM , 0.8 μM, 0.9 μM , 1.0 μM, 1.1 μM, 1.2 μM, 1.3 μM, 1.4 μM, 1.5 μM, 1.6 μM, 1.7 μM, 1.8 μM, 1.9 μM), Tris-HCl buffer (50 mM, pH 8.5), 30 ℃, λ_{ex} = 335 nm, λ_{em} = 494 nm, slit: 10/10 nm. Peak areas was integrated and fitted to a linear by origin, estimated equation y = 4077.326 x + 187.627 (r^2 = 0.998).

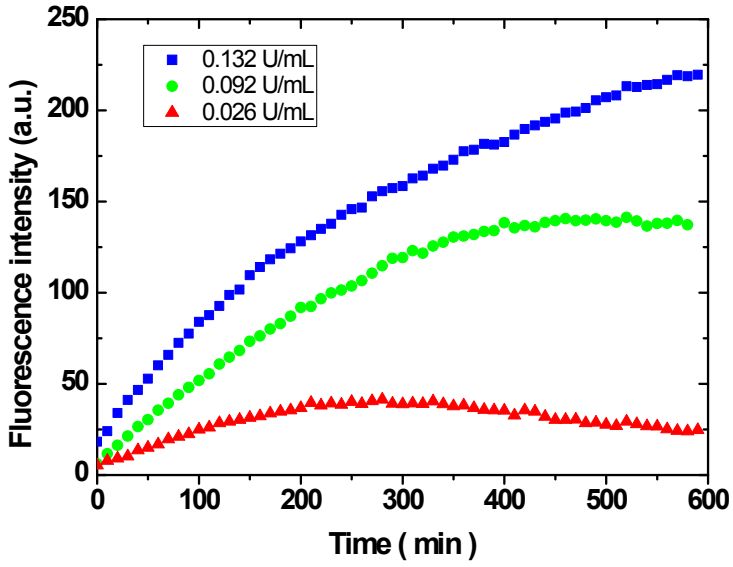


Fig. S4 Time course of fluorescence spectra of probe in presence of with PAL (0.026 U/mL, 0.092 U/mL, 0.132 U/mL) in 50 mM Tris-HCl buffer (50 mM, pH 8.5) at 494 nm versus reaction time, 30 ℃, λ_{ex} = 335 nm, slit: 10/10 nm.

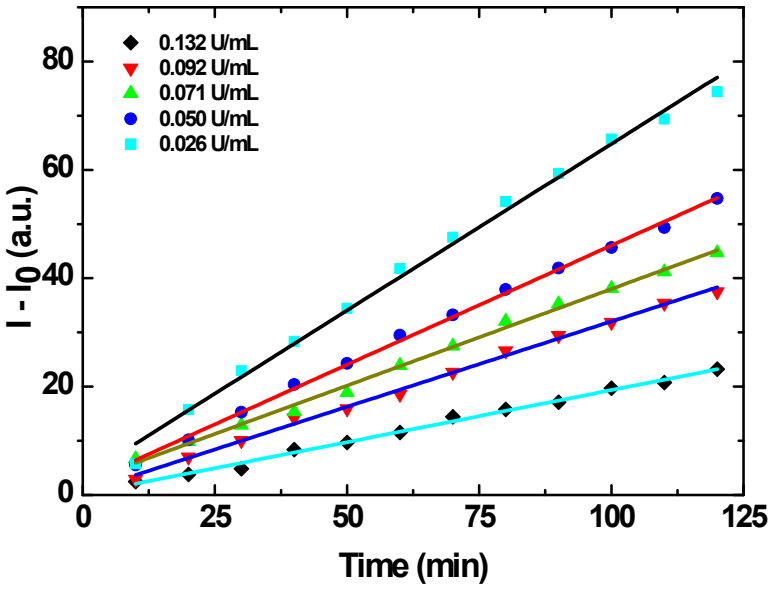


Fig. S5 Plot of the fluorescence intensity of 2a-LP with PAL versus reaction time. Conditions: PAL (0.026 U/mL, 0.050 U/mL, 0.071 U/mL, 0.092 U/mL, 0.132 U/mL), 2a-LP (5 mM), Tris-HCl buffer (50 mM, pH 8.5), 30 ℃, λ_{ex}= 335 nm, λ_{em}= 494 nm, slit: 10/10 nm. Each of the increased fluorescence were fitted to linear, estimated

equation (from blue line to black) y = 0.192 x + 0.158 (r^2 = 0.994), y = 0.315 x + 0.541 (r^2 = 0.997), y = 0.357 x + 2.365 (r^2 = 0.996), y = 0.440 x + 2.0514 (r^2 = 0.998), y = 0.614 x + 3.342 (r^2 = 0.993).

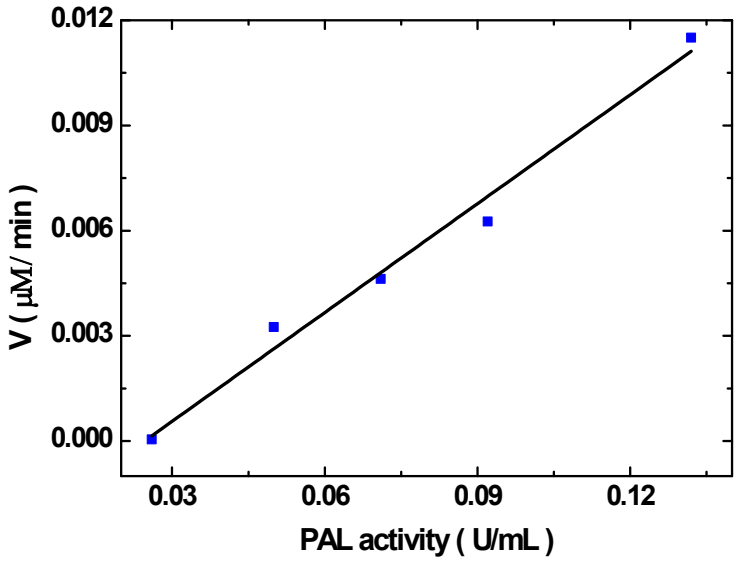


Fig. S6 Fluorimetric assay of PAL and 2a-LP. Conditions: enzyme (0.026-0.132 U/mL), 3e (5 Mm), Tris-HCl buffer (50 mM, pH 8.5), 30ºC, λ_{ex} = 335 nm, λ_{em} = 494 nm. Initial velocity values were calculated from the respective reaction time course by measuring the fluorescence signal at beginning 1 h. Initial velocity values were fitted to a linear equation with Origin, y = 0.103 x – 0.00254 (r^2 = 0.980).

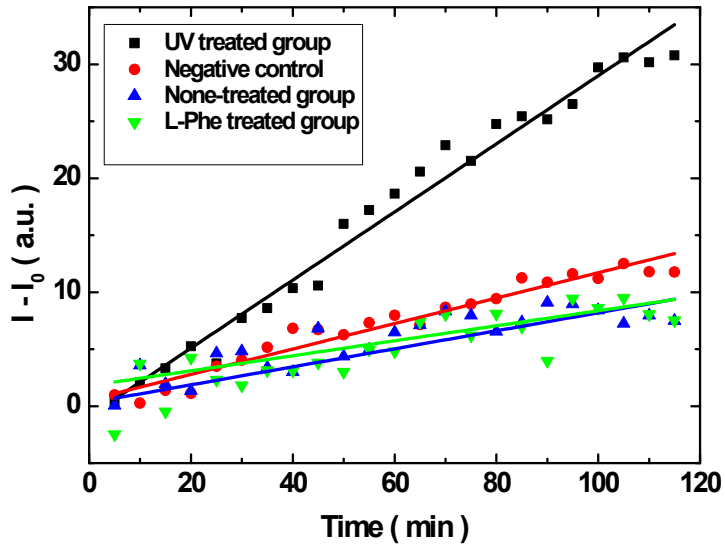


Fig. S7 Time course of fluorescence spectra of probe in presence of the crude PAL

extract. Conditions: 2a-LP (5 μM), λex = 315 nm, slit = 10/10 nm. UV treated group (black line: the crude enzyme extract solution from was from UV-A pretreatment tomato), Negative control (red line: the crude enzyme extract solution from none pretreatment tomato without addition of probe) None pretreatment (green line: the crude enzyme extract solution from none pretreatment tomato), L-Phe treated group (blue line: the crude enzyme extract solution from was from UV pretreatment tomato with addition 5μM L-Phe).