Supporting Information

Transformation of streptonigrin to streptonigrone: flavin reductase-mediated flavin-catalyzed concomitant oxidative decarboxylation of picolinic acid derivatives

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1. Materials and Methods

1.1 General Information

All commercially available compounds were purchased from Sigma-Aldrich, Aladdin, and TCI (Shanghai). High Performance Liquid Chromatography (HPLC) analysis of reactions was conducted with an Agilent 1200 series liquid chromatography (Agilent technologies) coupled with ZORBAX SB-C18 (Agilent 5 μm 4.6 × 150 mm) column at the flow rate of 0.3 mL min^{-1}. High resolution MS was performed using Agilent 6530 Accurate-Mass Q-TOF LC-MS spectrometer coupled with an Agilent HPLC 1200 series with ZORBAX SB-C18 (Agilent, 5 μM, 4.6 × 150 mm) column at the flow rate of 0.3 mL min^{-1}. NMR spectra were recorded on a Bruker AV-500 MHz NMR spectrometer with tetramethylsilane (TMS, 0.0 ppm) as the internal standard. Column chromatography was performed using silica gel (80−100 mesh; 200−300 mesh; 300−400 mesh; Haiyang Silica gel development Inc. Qingdao, China), reverse-phase silica gel (C18, 50 μm, YMC, Japan), and molecular sieve (Sephadex LH-20, GE, USA).

PCR amplifications were performed on a Veriti thermal cycler (Applied Biosystems, Carslbad, CA) using KOD-plus high fidelity DNA polymerase which was purchased from Takara Co. Ltd Company (Dalian, China). Restriction enzymes and DNA ligase were purchased from Fermentas (Thermo Fisher Scientific Inc, MA, USA) or NEB companies (Gene, England). Xanthine oxidase was purchased from Sigma-Aldrich. Superoxide dismutase was purchased from Aladdin. Gel extraction kits were purchased from Omega Bio-tek (GA, USA). Unless otherwise stated, other biochemicals and chemicals were purchased from standard commercial sources. All DNA manipulations in *E. coli* and *Streptomyces* were performed according to standard procedures. DNA sequencing was performed in Jie Li Biotech Co. Ltd. (Shanghai, China). All primers used in this work were synthesized by Sangong Biotech Co. Ltd. Company (Shanghai, China).

Expression and purification of StnD, $StnH_2$, $StnH_3$, flavin reductase (Fre)

PCR-amplified products of the genes *stnD*, *$stnH_2$*, and *$stnH_3$*, were digested using

NdeI-XhoI. The resulting fragments were cloned into pET28a digested with the same restriction enzymes to yield the expression plasmid pLS1150-*stnD*, pLS1151-*stnH*$_2$ and pLS1152-*stnH*$_3$. The *E. coli* BL21 (DE3) pLysS cells harboring the expression plasmid were cultured at 37 ℃ and 220 rpm in LBBS medium (10g/L Tryptone, 5g/L Yeast Extract, 5g/L NaCl, 182g/L Sorbitol, 0.3g/L Betaine) supplemented with 50 μg mL^{-1} kanamycin and 25 μg mL^{-1} chloramphenicol (final concentration) to OD_{600} about 0.6. The culture was then incubated on ice for 10 min before addition of 0.2 mM (final concentration) IPTG to induce protein expression. The expression of Fre followed the procedures provided before.[1] The cells were further cultured at 16 ℃ for 24 h, and then were harvested by centrifugation (3500 rpm, 15 min, 4 ℃), re-suspended with buffer A (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, and 10% glycerol) and lysed by sonication on ice for 40 min. Cellular debris was removed by centrifugation (12,500 rpm, 45 min, 4 ℃), and the supernatant was loaded onto nickel-affinity chromatography columns. The protein was eluted with increasing gradient of buffer B (500 mM imidazole in buffer A). Purified proteins were concentrated and exchanged into buffer C (50mM Tris-HCl, pH 8.0, 50 mM NaCl, and 5% glycerol) with the centriprep filters (Amicon). The protein purity was judged by SDS-PAGE analysis and was stored in buffer C at -80 ℃ (Figure S1). Protein concentration was determined by Bradford assay using bovine serum albumin as a standard.

Procedures for Purification of compounds

(1) Fermentation and purification of streptonigrin (**1**)

The *Streptomyces flocculus* CGMCC 4.1223 wild-type strain was spread on agar plates which was composed of 20 g of soybean meal, 20 g of mannitol, 20 g of agar per liter (pH 7.0-7.5), and then incubated at 30 ℃ for sporulation and growth. Approximately 1 cm^2 of the sporulated agar of *S. flocculus* was cut, chopped, and inoculated into the seed medium (25 mL in 100 mL flask) which was composed of 3% TSB and 0.5% yeast extract per liter. After incubation at 30 ℃ and 220 rpm for 36 h, 1 mL of the seed culture broth was transferred into the fermentation medium (100 mL in 500 mL flask), which was composed of 2.5% glucose, 1.5% soybean meal, 0.5%

NaCl, 0.05% KCl, 0.3% K_2HPO_4, 0.025% $MgSO_4 \cdot 7H_2O$, and 0.3% $Na_2HPO_4 \cdot 12H_2O$. Further incubation was carried out at 30 ℃ and 220 rpm for 7 days. After finishing the fermentation, the broths were harvested by centrifugation and extracted with ethyl acetate. In order to purify enough streptonigrin, 21 L fermentation broths were extracted with ethyl acetate for three times. After evaporation, the crude extracts were subjected to silica gel column chromatography and eluted with petroleum ether/EtOAc (10:1-1:3), 100% EtOAc, EtOAc/CH_3OH (10:1-1:2), and 100% CH_3OH to give 36 fractions. Each fraction was monitored by HPLC with the authorized compound as the reference. The fractions containing streptonigrin were collected and separately subjected to the reverse phase silica gel column chromatography. The streptonigrin was eluted with H_2O/CH_3OH (70:30-0:100). All the fractions were monitored by HPLC. The fractions containing streptonigrin were collected and concentrated. The resultant residues were re-dissolved in CH_3OH, loaded on Sephadex LH-20 column, and eluted with CH_3OH. The fractions containing the pure streptonigrin were collected and dried.

Streptonigrin **1** (77 mg) was dark red powder. Its molecular formula was established as $C_{25}H_{22}N_4O_8$ based on the HR-ESI-MS (*m/z* 507.1506, calcd. 507.1510 $[M + H]^+$).

The substrates including **1b**, **1c**, **1d**, and **3** were obtained by F Xu in the previous work.[2]

(2) Purification of product **2** (streptonigrone)

The reaction mixture, comprising 10 μM of FAD, 100 μM of streptonigrin (**1**), 2 μM of flavin reductase, 5 mM of NADH, 50 mM PBS buffer (pH 7.5), in a final volume of 100 mL and 50 mL, was incubated at 25 ℃. The reaction was monitored by HPLC analysis under the following conditions: solvent A [1‰ (vol/vol) formic acid in ddH_2O] and solvent B [1‰ (vol/vol) formic acid in Acetinitrile (CAN)]; the mobile-phase composition was 10%-100% B at 0-25 min, 100% at 30 min, 100%-10% B at 30-31 min, 10% B at 31-41 min at the flow rate of 0.3 mL min^{-1} with detection at 210 nm, 375 nm, and 420 nm. After finishing transformation, the mixture

was extracted with EtOAc for three times. After evaporation, the extracts were subjected to the reverse phase column chromatography and eluted with H_2O /CH_3OH (70:30-0:100). The last 20 fractions with greenish yellow were monitored by HPLC. The fractions containing **2** were collected and concentrated. The resultant residues were re-dissolved in CH_3OH, loaded on Sephadex LH-20 column, and eluted with CH_3OH. The fractions containing **2** were collected and dried.

2 (22 mg) was obtained. The molecular formula were established as $C_{24}H_{22}N_4O_7$ based on the HR-ESI-MS (*m/z* 479.1561 $[M+H]^+$) and the NMR spectra (Figure S3).

General Procedure for flavin-catalyzed oxidative decarboxylation

The flavin-catalyzed reactions were carried out at 25 °C for 10 min in a 100 μL reaction mixture. The reaction mixture contained 100 μM of substrate, 2 μM of flavin reductase, 5 mM of NADH, 10 μM of FAD, 50 mM PBS buffer (pH 7.5). Reactions without FAD were used as negative controls. Due to the reaction was initiated by adding flavin reductase and 4α-hydroperoxyflavin (FIOOH) easily eliminates H_2O_2 to regenerate the oxidized flavin, flavin reductase was the last component adding to the reaction mixture. After 10 minutes' standing, the mixture turned into greenish yellow from top to bottom. Reactions were quenched using 200 μL of ethyl acetate for extraction twice. After removing ethyl acetate by vacuum evaporation, the residues were dissolved in 40 μL of methanol and subjected to HPLC analysis or LC-HR-MS in positive mode.

When streptonigrin analogues (**1a-d**, and **3**) were used as the substrates to test the scope of the reaction, the reactions were quenched by the extraction of ethyl acetate. The conversion was measured by HPLC analysis under the following conditions: 10%-100% B at 0 -25 min, 100% at 30 min, 100%-10% B at 30-31 min, 10% B at 31-41 min at the flow rate of 0.3 mL min^{-1} with detection at 210 nm, 375 nm, and 420 nm. The products were determined by High-resolution LC-MS (Figure S10-S14).

When the substrates were picolinic acids, an equal volume of methanol was added into each mixture to terminate the reaction. After removal of the denatured

protein (flavin reduactase) by centrifugation, the reaction mixtures were subjected to HPLC analysis or LC-ESI-MS in positive mode. HPLC analyses were performed under the following conditions: 2% to 40% B (0-20 min); 40% -95% B (20-25 min); 95% B (25-30 min); 95% to 2% B (30-31 min); 2% B (31-41 min) at the flow rate 0.3 mL $min^{-1}$and UV detection at 210 nm, 280 nm, 300 nm, 320 nm. The products were confirmed by high-resolution MS and comparison with the standards (Figure S15-S20).

Procedure for the time-dependent reactions for detection of the concentration change of 1, 2, NAD^+ and NADH

In order to determine the concentration change of **1**, **2**, NAD^+ and NADH, the reactions conatining 10 μM FAD, 5 mM NADH, 2 μM flavin reductase, 100 μM streptonigrin and 50 mM PBS buffer (pH 7.5) in a total volume of 100 μL were carried out at 25°C in triplicate. Flavin reductase was the last component adding to the reaction mixture. After mixing them and standing a range of time (2, 4, 6, 8, 10 min), reactions were quenched using 200 μL of MeOH. After removing water and MeOH by vacuum evaporation, the residues were dissolved in 40 μL of methanol and subjected to HPLC analysis. To quantify the amount of them from each reaction, a standard curve based on the HPLC peak area correlated with the known amount of the authentic standards was first generated. HPLC analyses were performed under the following conditions: 2% to 40% B (0-20 min); 40% -95% B (20-25 min); 95% B (25-30 min); 95% to 2% B (30-31 min); 2% B (31-41 min) at the flow rate 0.3 mL $min^{-1}$and UV detection at 260 nm, 340 nm, 375 nm, 420 nm (Figure S8).

Procedure for xanthine oxidase/hypoxanthine system generation superoxide

Reactions all performed at 25 °C, in 50mM phosphate buffer containing 100 μM streptonigrin or 250 μM *p*-nitroblue tetrazolium, at pH7.5. Superoxide was generated from hypoxanthine (200 μM) and xanthine oxidase (0.05 U/mL). Reactions were quenched using 200 μL of ethyl acetate for extraction twice. After removing ethyl acetate by vacuum evaporation, the residues were dissolved in 40 μL of methanol and subjected to HPLC analysis. The reaction was monitored by HPLC analysis under the

following conditions: solvent A [1‰ (vol/vol) formic acid in ddH_2O] and solvent B [1‰ (vol/vol) formic acid in Acetinitrile (CAN)]; the mobile-phase composition was 10%-100% B at 0-25 min, 100% at 30 min, 100%-10% B at 30-31 min, 10% B at 31-41 min at the flow rate of 0.3 mL min^{-1} with detection at 210 nm, 375 nm, and 420 nm (Figure S25).

Reference

1. Lin, S.; Van Lanen, S. G.; Shen, B., *J. Am. Chem. Soc.* **2007,** *129*, 12432-12438.

2. Xu, F.; Kong, D. K.; He, X. Y.; Zhang, Z.; Han, M.; Xie, X. Q.; Wang, P.; Cheng, H. R.; Tao, M. F.; Zhang, L. P.; Deng, Z. X.; Lin, S. J., *J. Am. Chem. Soc.* **2013,** *135*, 1739-1748.

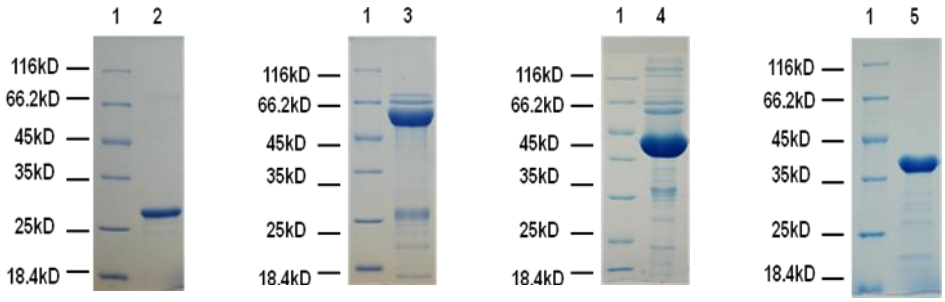

Figure S1. Characterization of recombinant proteins: lane 1, low-range molecular weight standards; lane 2, Fre; lane 3, StnD; lane 4, $StnH_2$; lane 5, $StnH_3$.

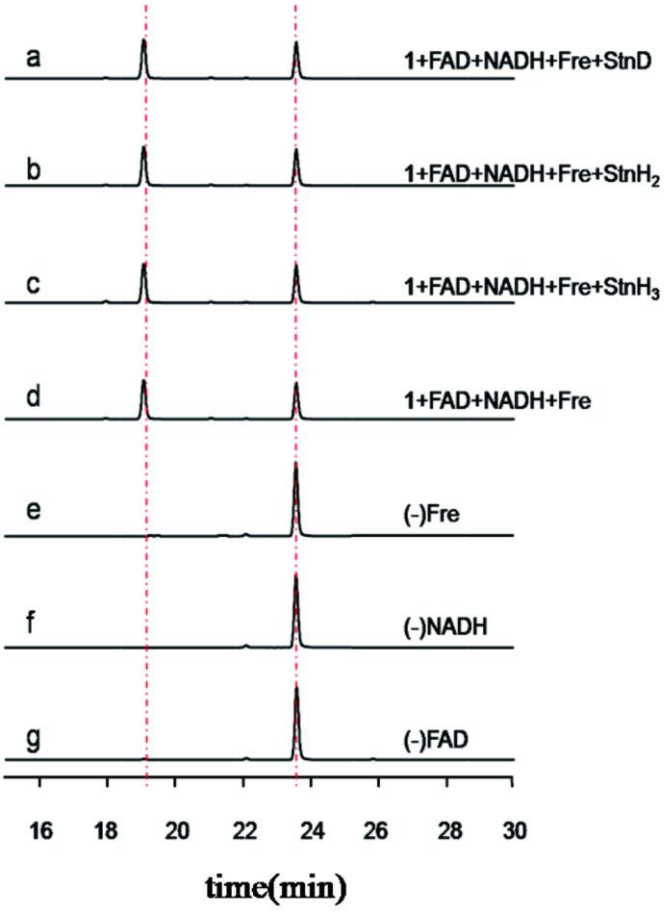

Figure S2. Cofactor dropout assays. (a) complete assay with 30 μM StnD; (b) complete assay with 30 μM $StnH_2$; (c) complete assay with 30 μM $StnH_3$; (d) complete assay; (e) complete assay minus flavin reductase; (f) complete assay minus NADH; (g) complete assay minus FAD. Full reaction mixture contained **1** (100 μM), flavin reductase Fre (2 μM), FAD (10 μM) and NADH (5 mM). StnD, $StnH_2$ and $StnH_3$ are flavoproteins in the streptonigrin biosynthetic pathway. HPLC was monitored at 375 nm.

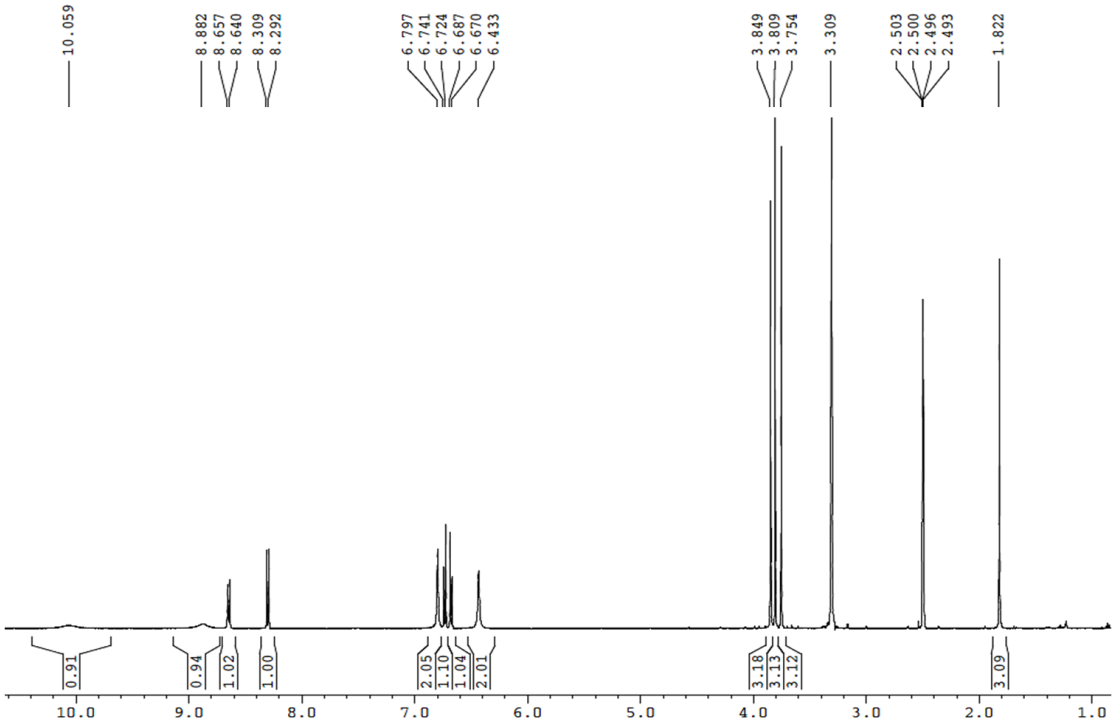

Figure S3A. ^{1}H NMR spectrum of **2** in DMSO-d_6 (500 MHz).

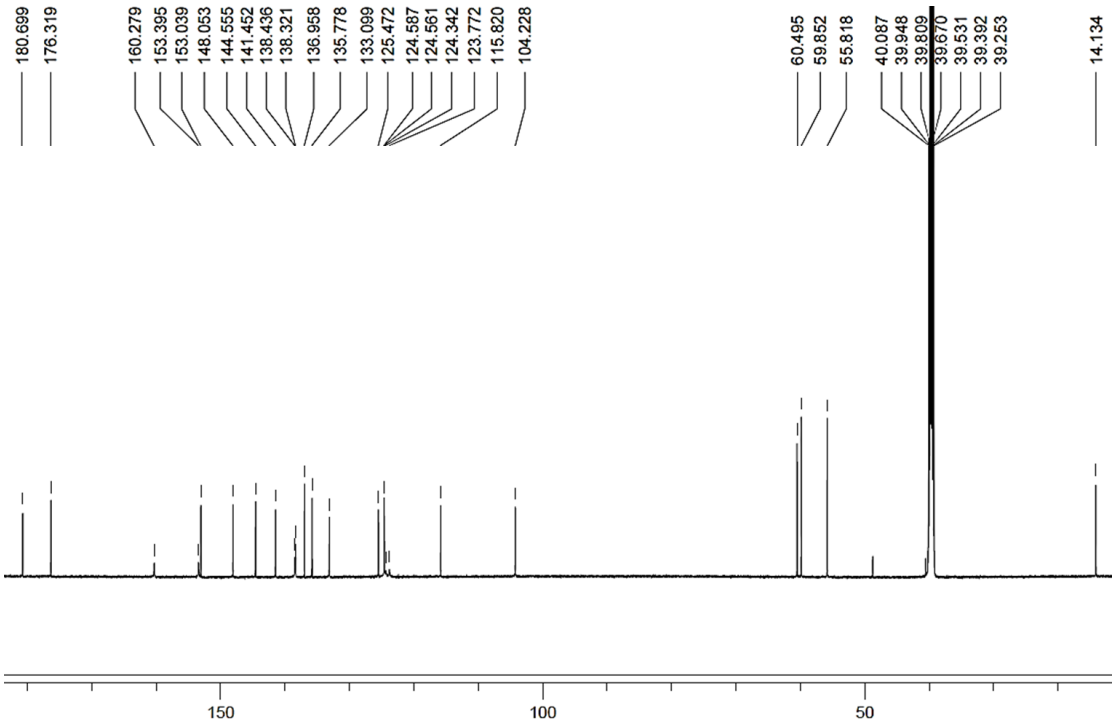

Figure S3B. ^{13}C NMR spectrum of **2** in DMSO-d_6 (150 MHz).

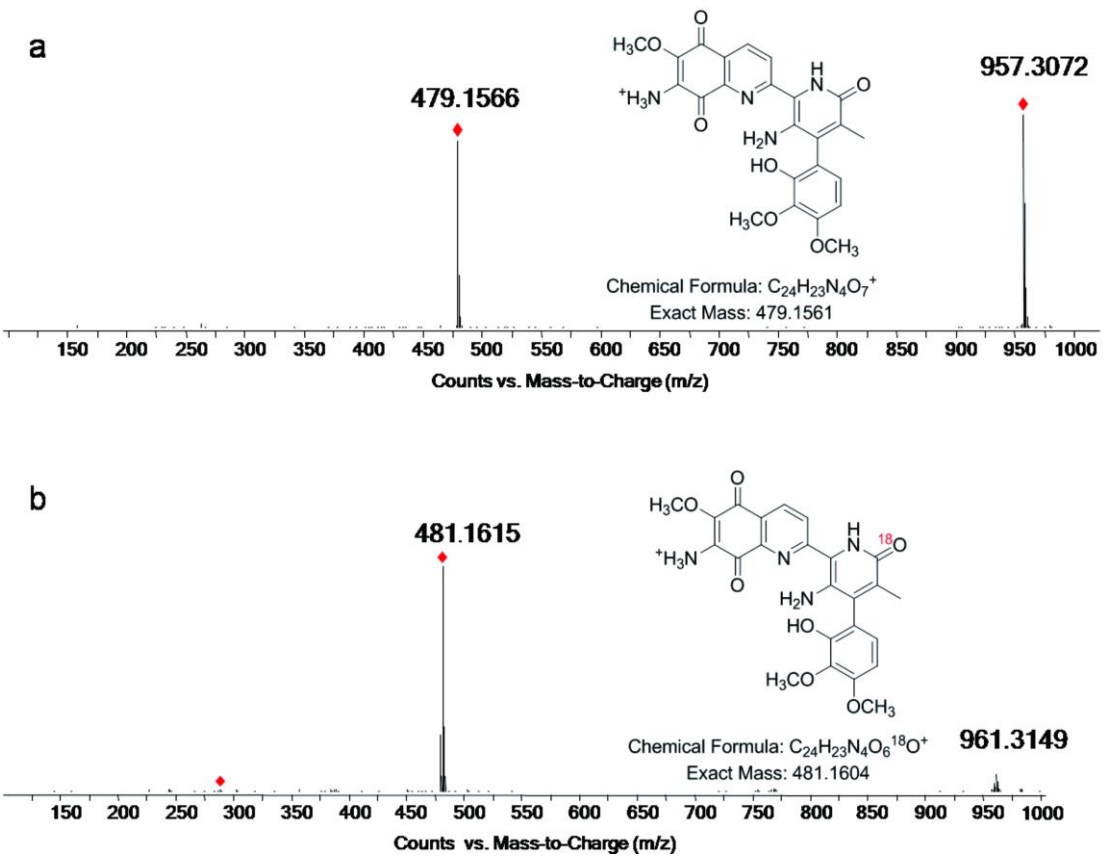

Figure S4. HR-ESI-MS profiles of **2**. (a) The HR-MS of **2** from the reaction that was carried out with air, 957.3072 $[2M + H]^+$ (calc.: 957.3050); (b) The HR-MS of **2** from the reaction that was carried out with $^{18}O_2$.

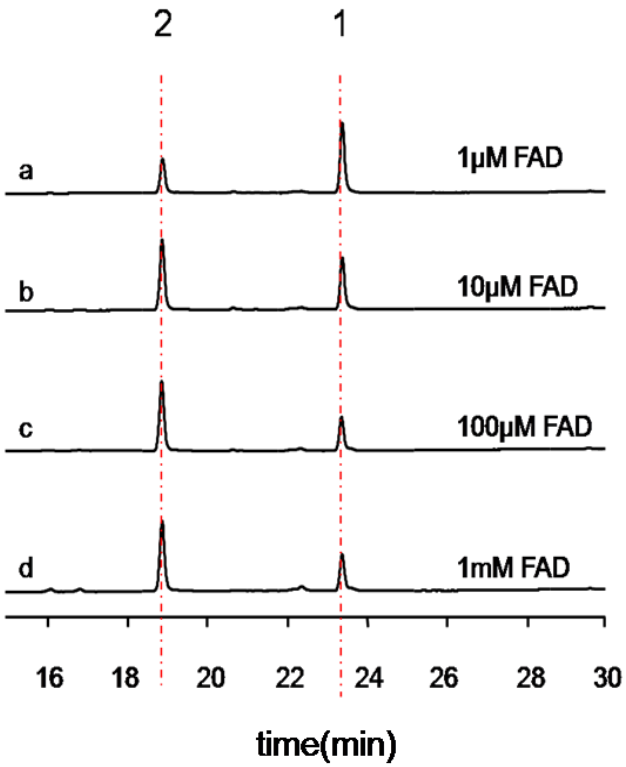

Figure S5. Effect of FAD concentration on the reaction. (a) 1 μM; (b) 10 μM; (c) 100 μM; (d) 1 mM. The reactions were monitored by HPLC analysis at 375 nm.

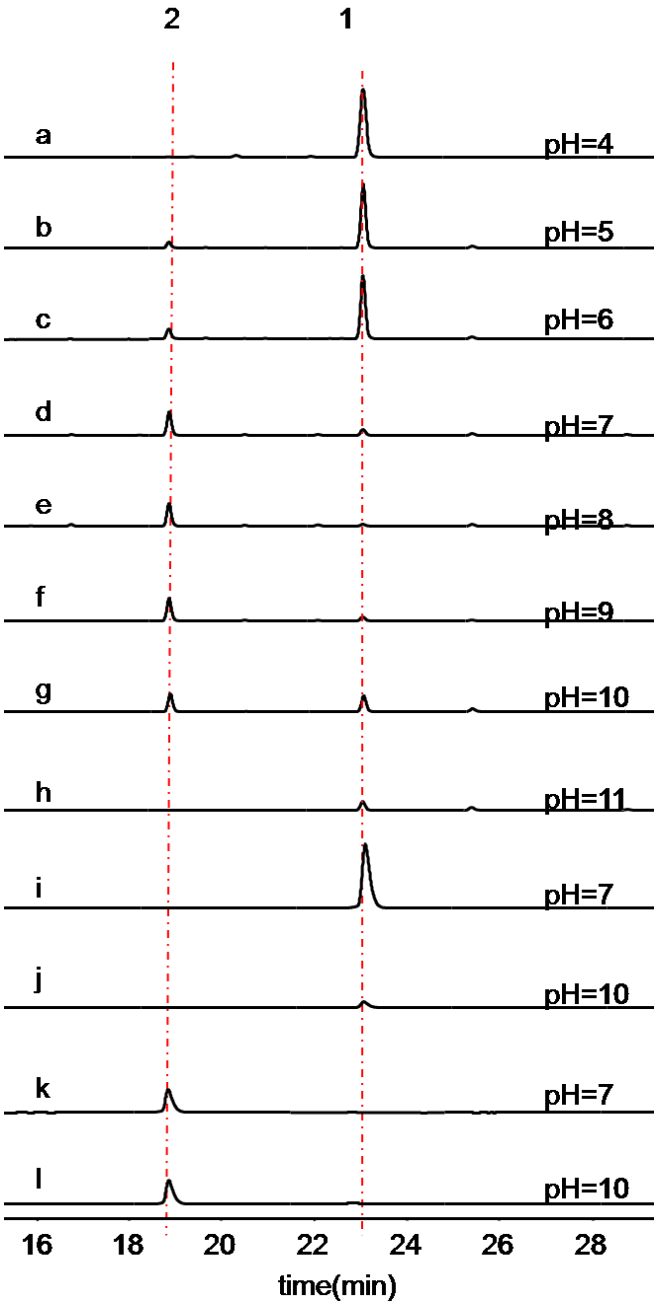

Figure S6. The effect of the buffer pH on the reaction. (a) citric acid/Na_2HPO_4 buffer (pH 4.0); (b) citric acid/Na_2HPO_4 buffer (pH 5.0); (c) citric acid/Na_2HPO_4 buffer (pH 6.0); (d) NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0); (e) boric acid/borax buffer (pH 8.0); (f) boric acid/borax buffer (pH 9.0); (g) borax/NaOH buffer (pH 10.0); (h) Na_2HPO_4/Na_3PO_4 buffer (pH 11.0); (i) streptonigrin in NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0); (j) streptonigrin in borax/NaOH buffer (pH 10.0); (k) streptonigrone in NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0); (l) streptonigrone in borax/NaOH buffer (pH 10.0). The pH dependence of the catalytic activity of FAD for the oxidation reaction was determined with 100 μM of streptonigrin, 2 μM of flavin reductase, 10 μM of FAD and 5 mM of NADH in 50 mM various buffers. The reactions were monitored by HPLC analysis at 375 nm.

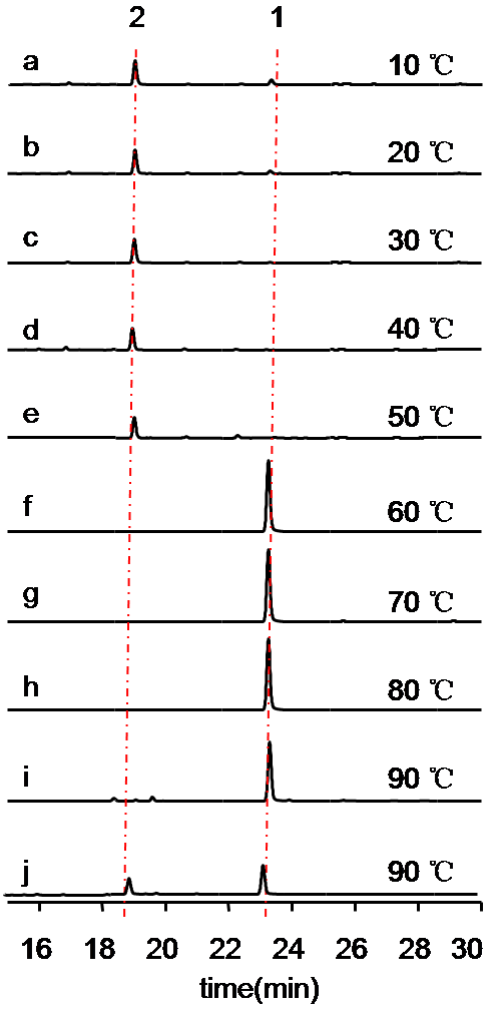

Figure S7. The effect of temperature on the reaction. (a-i) The reactions were carried out with flavin reductase; (j) The reaction was carried out with DTT. The reactions were monitored by HPLC analysis at 375 nm.

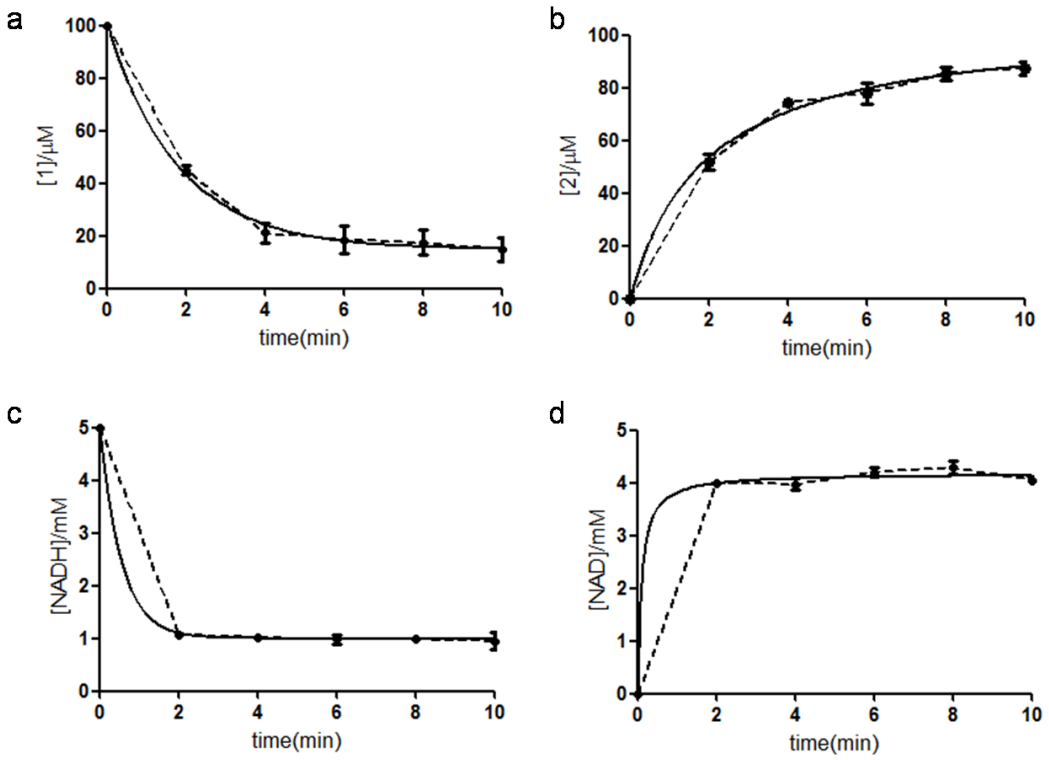

Figure S8. The concentration changes of **1**, **2**, NAD^+, NADH in the time-dependent reactions. Concentration changes of **1** (a), **2** (b), NADH (c), and NAD (d). Reactions were performed under the standard conditions in triplicates and monitored by HPLC with detection at 375 nm. The continuous lines represented the fitted curves and dash lines represented the experimental data.

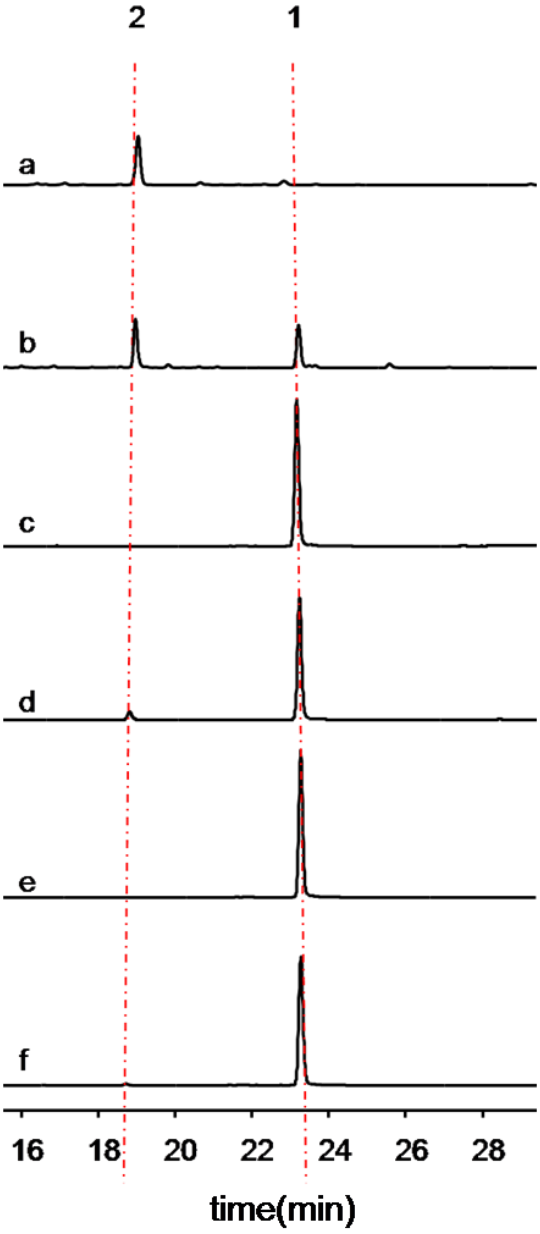

Figure S9. The effect of different reducing agents on the reaction. (a) 5 mM NADH (standard condition); (b) 1 mM DTT; (c) 1 mM Na_2S; (d) 10 mM Na_2S; (e) 1 mM $Na_2S_2O_4$ (sodium dithionite); (f) 10 mM $Na_2S_2O_4$. The reactions were monitored by HPLC analysis at 375 nm.

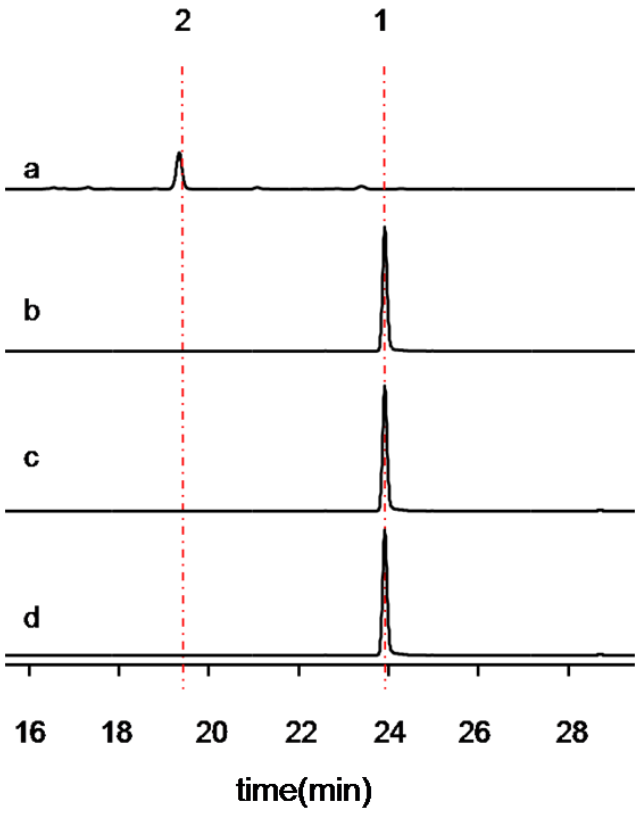

Figure S10. Different oxidants with or without FAD were tested in the reactions. (a) 10 μM FAD (standard condition); (b) 1 mM H_2O_2; (c) 1 mM Butyl hydroperoxide; (d) 1 mM 3-Chloroperbenzoic acid. The reactions were monitored by HPLC analysis at 375 nm.

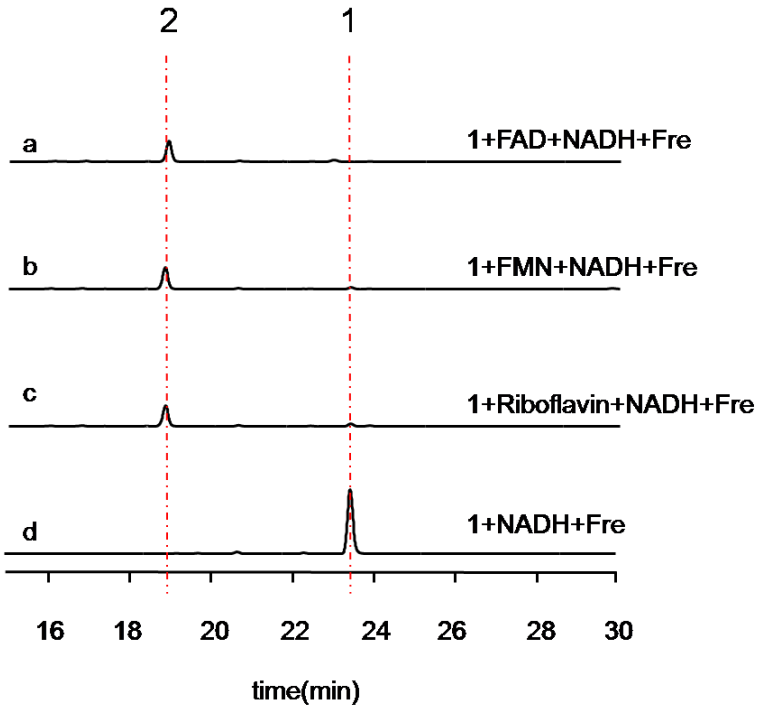

Figure S11. The reactions with different flavins. (a) FAD; (b) FMN; (c) Riboflavin; (d) without flavins. The reactions were monitored by HPLC analysis at 375 nm.

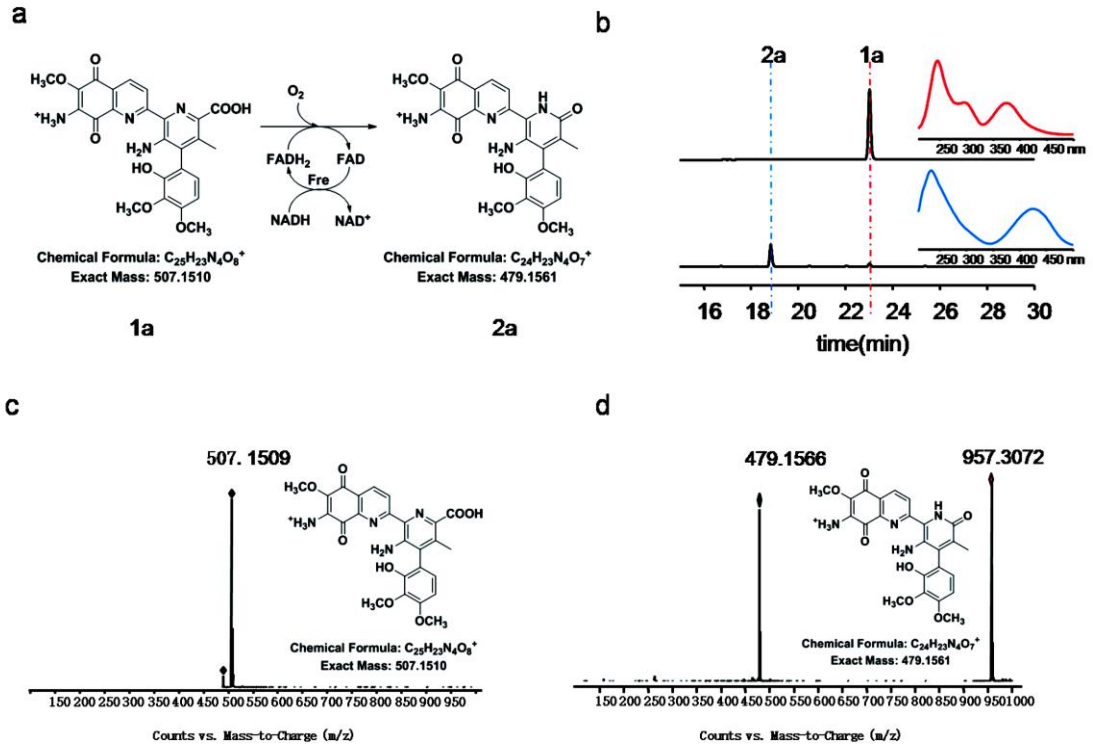

Figure S12. Characterization of the transformation of **1a** to **2a** catalyzed by FAD. a, Reaction equation; b, The HPLC profile of the reaction was monitored at 375 nm; c, High-resolution (HR) MS of **1a;** d, HR-MS of **2a**.

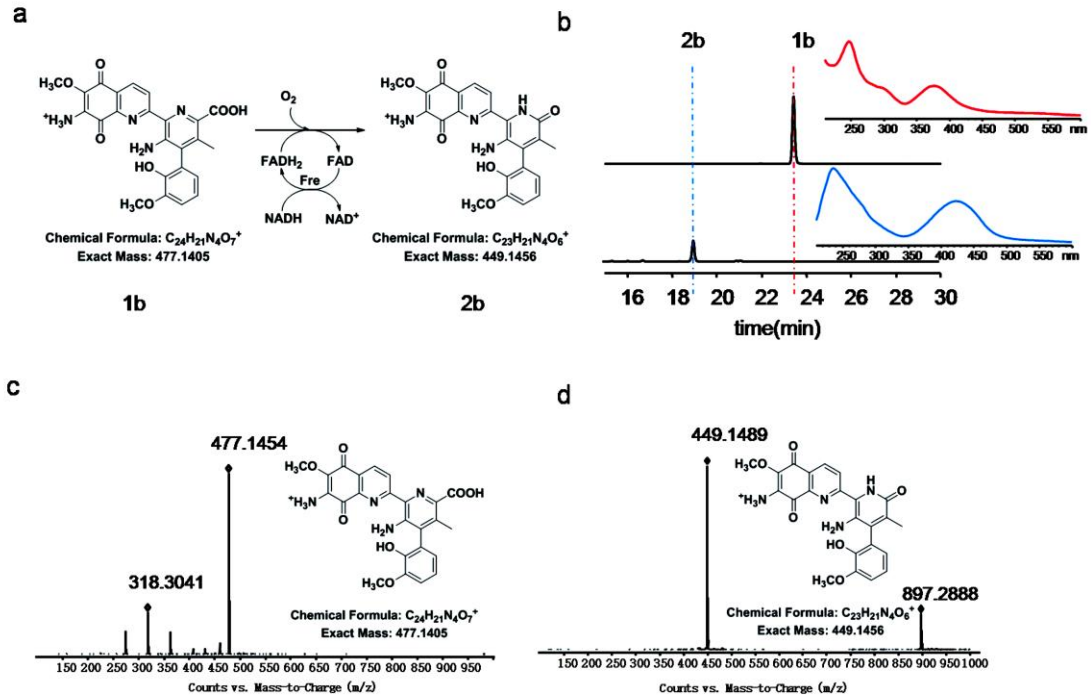

Figure S13. Characterization of the transformation of **1b** to **2b** catalyzed by FAD. a, Reaction equation; b, The HPLC profile of the reaction was monitored at 375 nm; c, HR-MS of **1b;** d, HR- MS of **2b**.

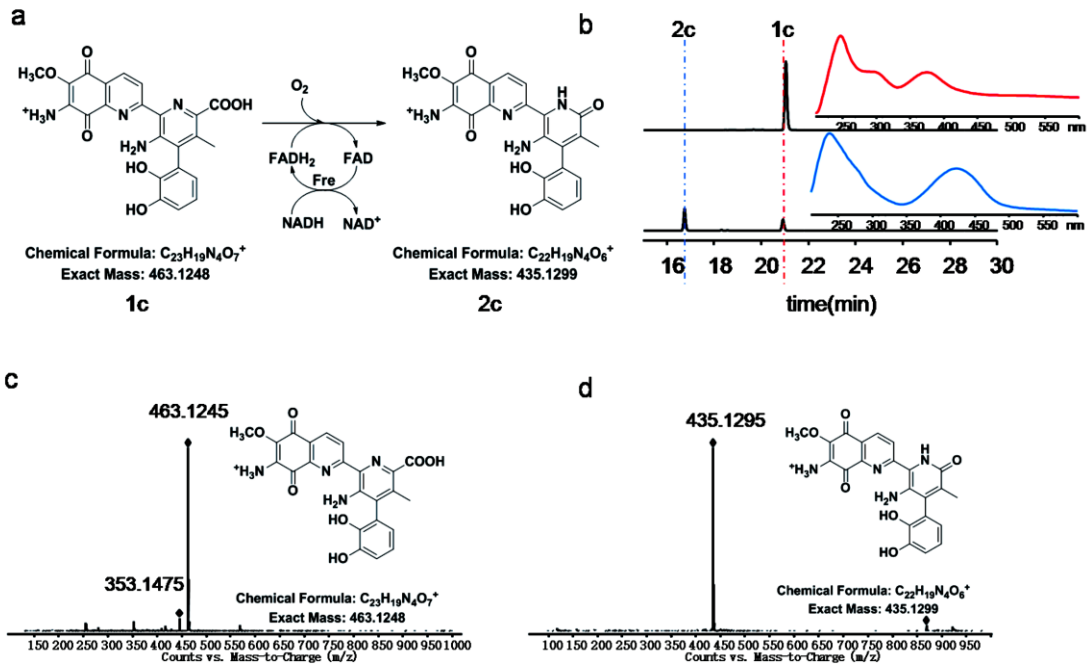

Figure S14. Characterization of the transformation of **1c** to **2c** catalyzed by FAD. a, Reaction equation; b, The HPLC profile of the reaction was monitored at 375 nm; c, HR-MS of **1c;** d, HR -MS of **2c**.

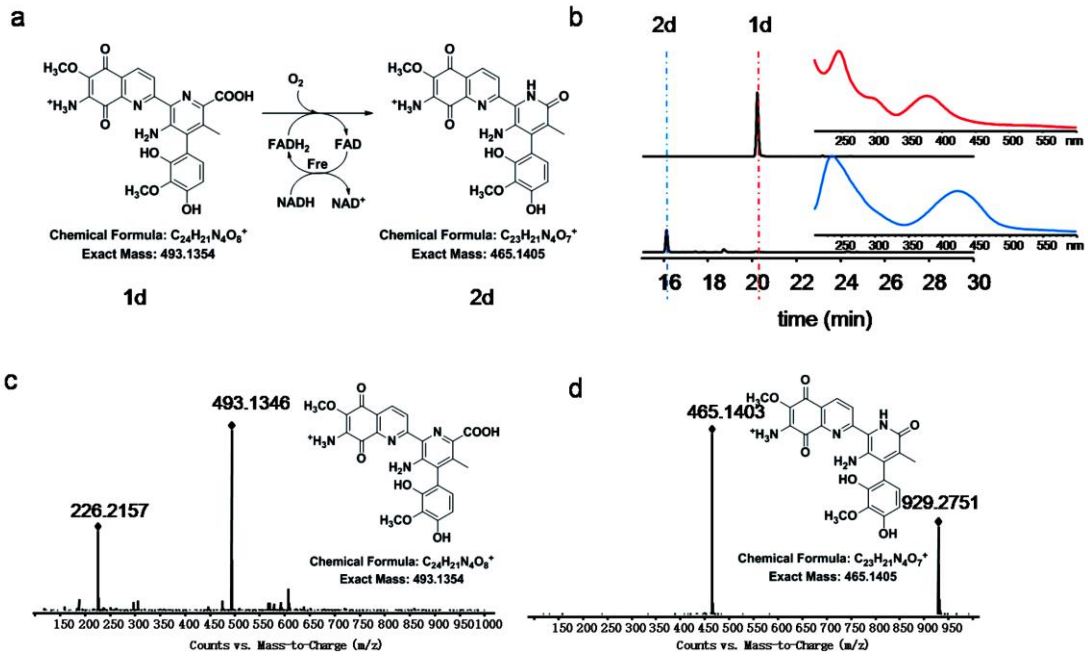

Figure S15. Characterization of the transformation of **1d** to **2d** catalyzed by FAD. a, Reaction equation; b, The HPLC profile of the reaction was monitored at 375 nm; c, HR-MS of **1d;** d, HR- MS of **2d**.

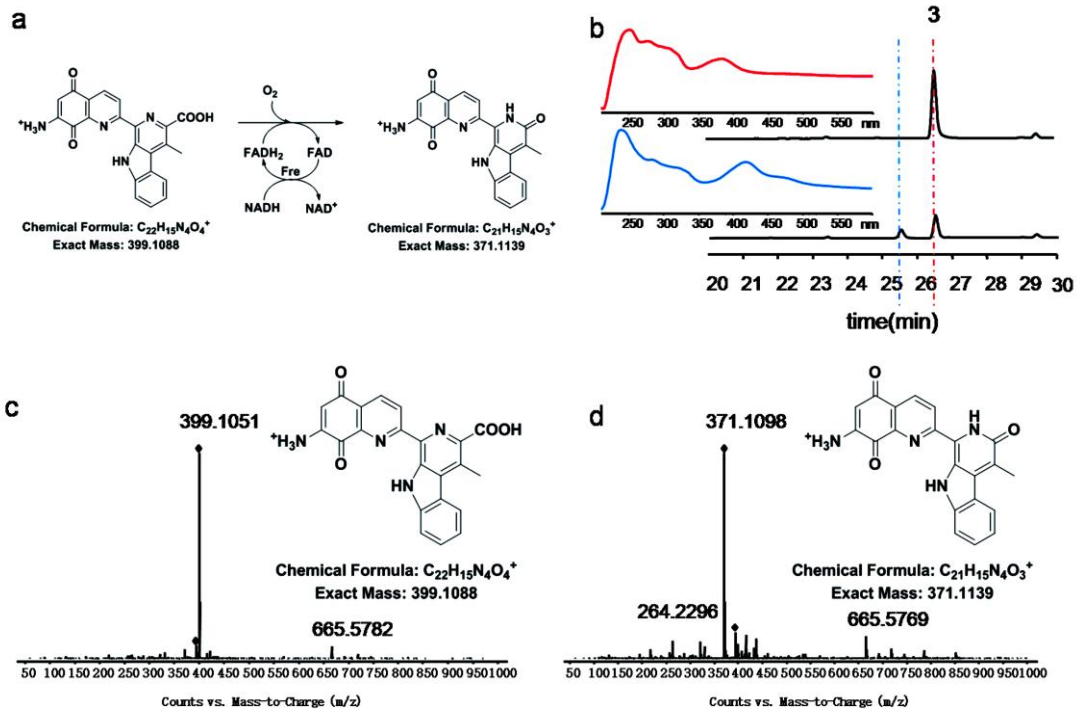

Figure S16. Characterization of the transformation of **3** catalyzed by FAD. a, Reaction equation; b, The HPLC profile of the reaction was monitored at 375 nm; c, HR-MS of **3;** d, HR-MS of the product.

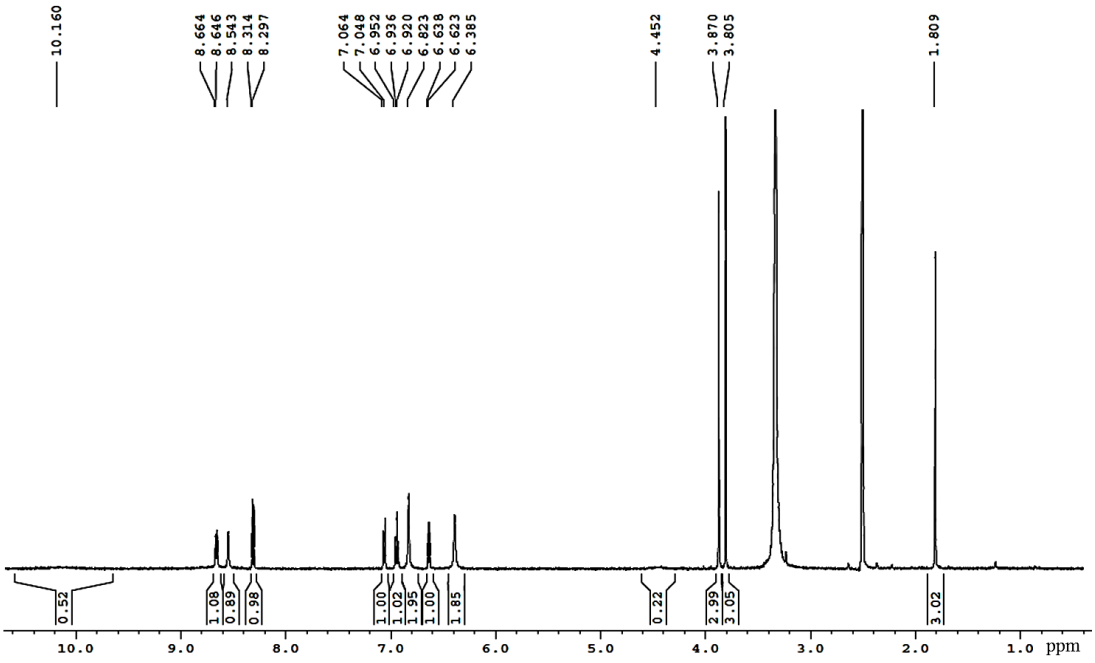

Figure S17. ^{1}H NMR spectrum of **2b** in DMSO-d_6 (500 MHz).

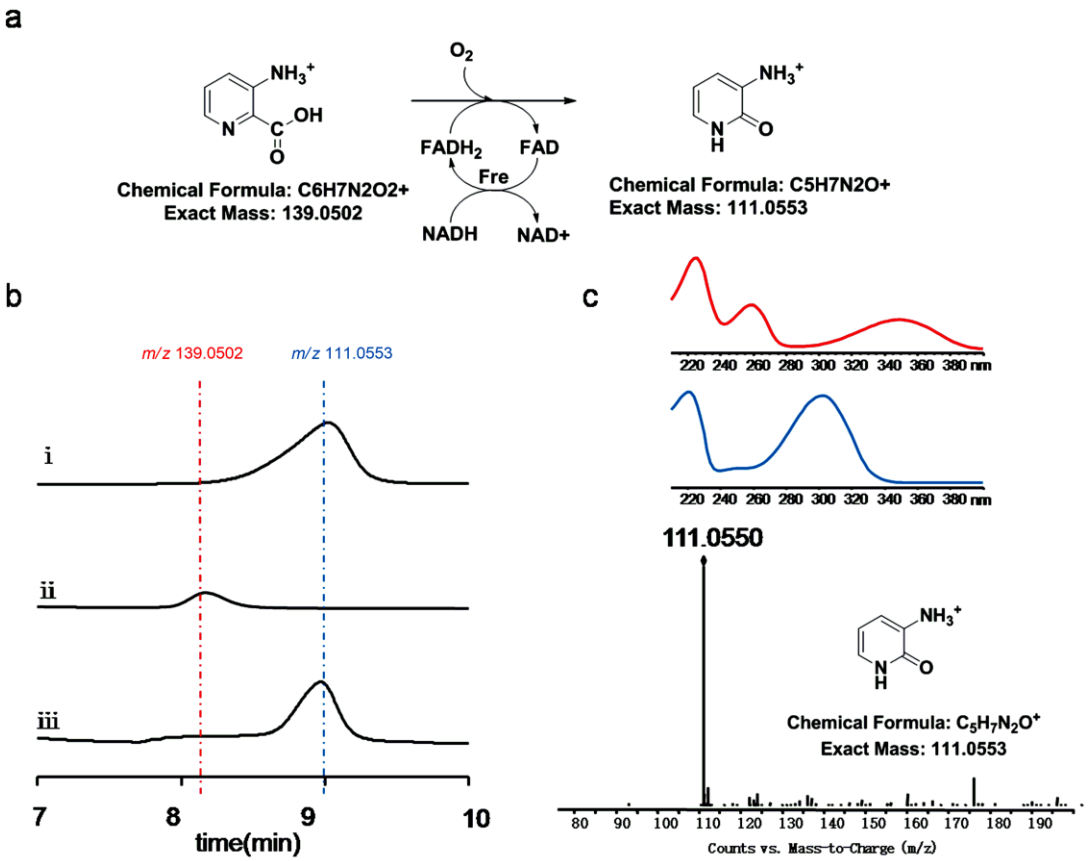

Figure S18. Characterization of the transformation of 3-aminopydridine-2-carboxylic acid **7** to 3-amino-2-pyridone catalyzed by FAD. a, Reaction equation; b, The HPLC profiles of the reaction were monitored at 300 nm, (i) authentic 3-amino-2-pyridone standard, (ii) control experiment (without FAD), (iii) standard reaction; c, UV spectra of **7** and product, and HR-MS of the product of the reaction with **7** as the substrate.

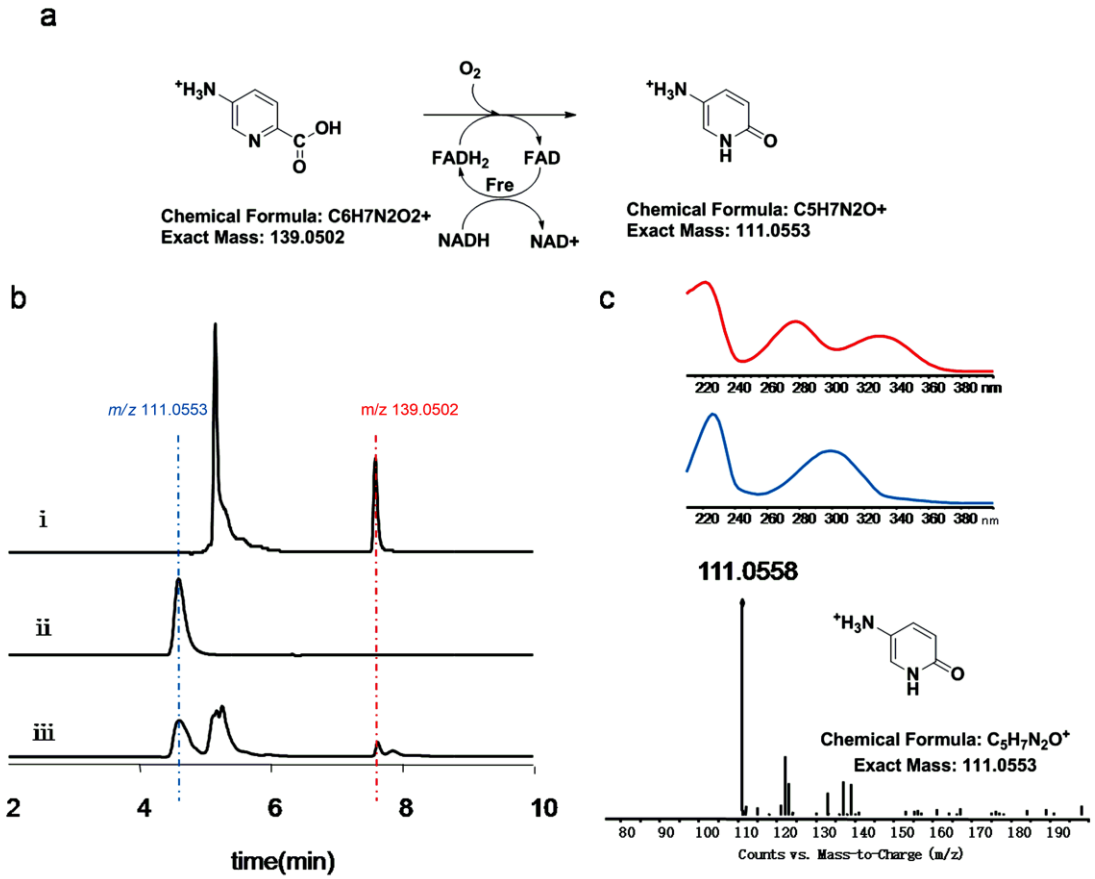

Figure S19. Characterization of the transformation of 5-aminopydridine-2-carboxylic acid **9** to 5-amino-2-pyridone catalyzed by FAD. a, Reaction equation; b, The HPLC profiles of the reaction were monitored at 300 nm, (i) control experiment (without FAD), (ii) authentic 5-amino-2-pyridone (standard), (iii) standard reaction: c, UV spectra of **9** and product, and HR-MS of the product of the reaction with **9** as the substrate.

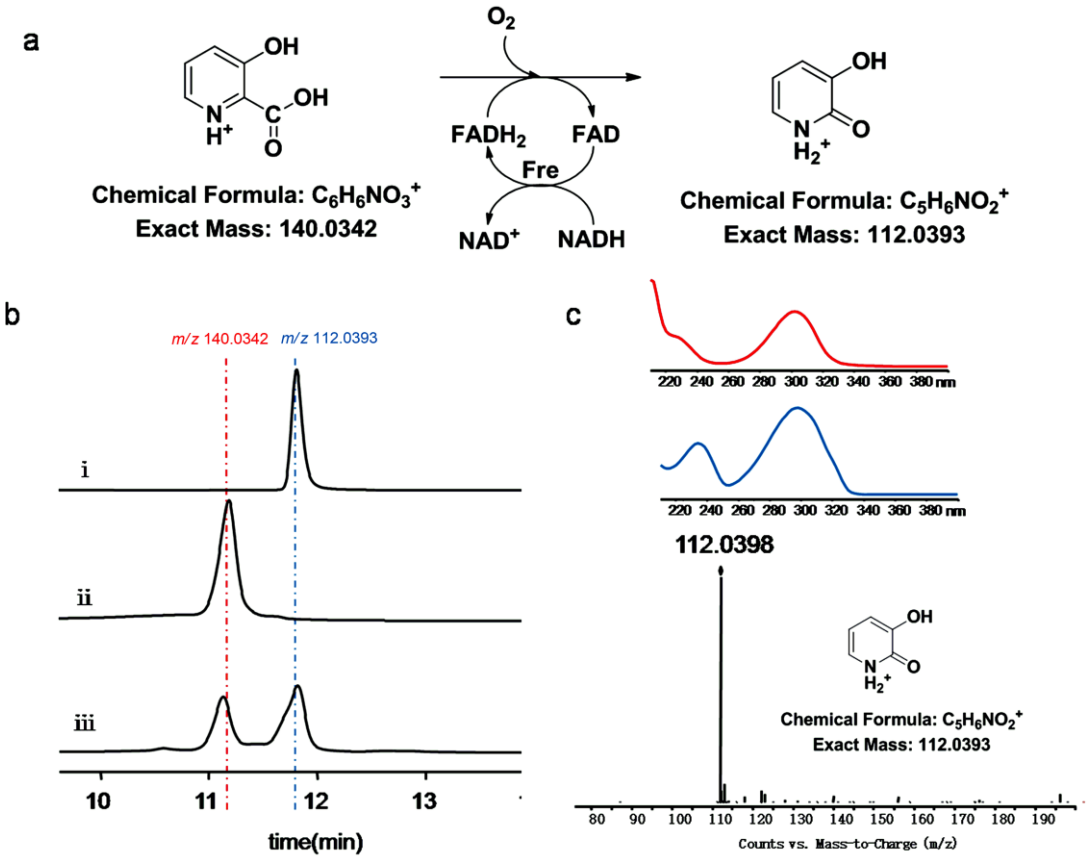

Figure S20. Characterization of the transformation of 3-hydroxypicolinic acid **11** to 3-hydroxy-2-pyridone catalyzed by FAD. a, Reaction equation; b, The HPLC profiles of the reaction were monitored at 300 nm, (i) authentic 3-hydroxy-2-pyridone (standard), (ii) control experiment (without FAD), (iii) standard reaction; c, UV spectra of **11** and product, and HR-MS of the product of the reaction with **11** as the substrate.

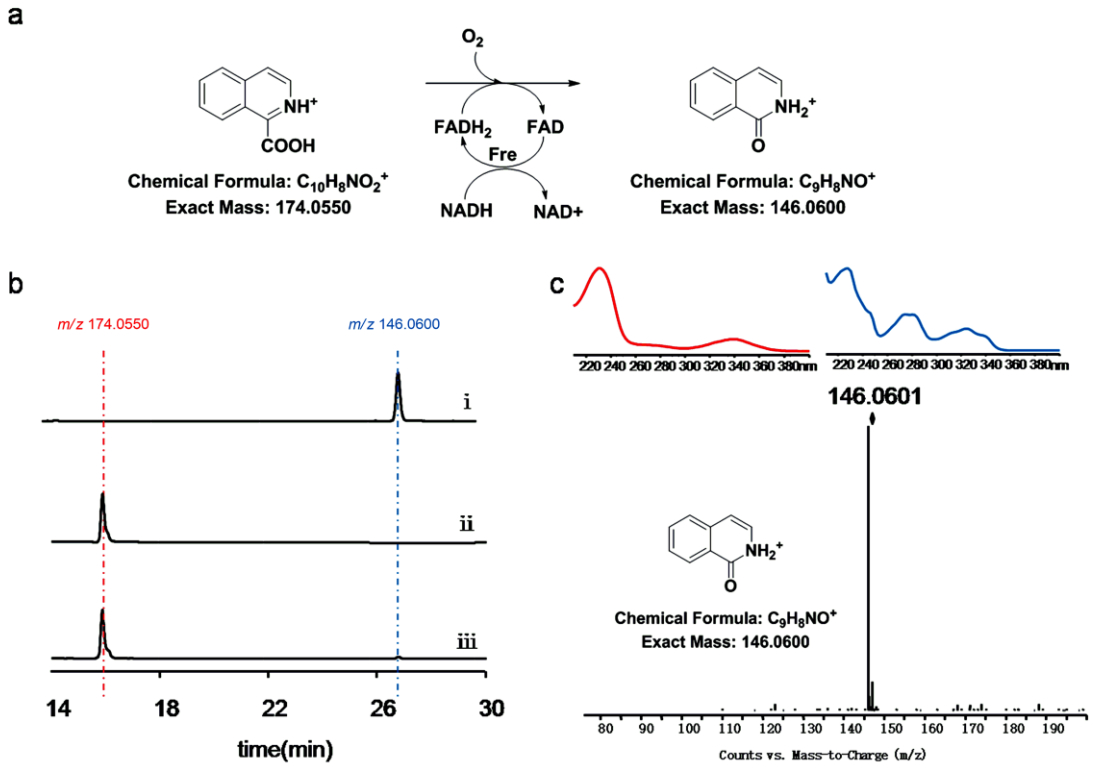

Figure S21. Characterization of the transformation of isoquinoline-1-carboxylic acid **20** to isocarbostyril catalyzed by FAD. a, Reaction equation; b, The HPLC profiles of the reaction were monitored at 300 nm, (i) authentic isocarbostyril (standard), (ii) control experiment (without FAD), (iii) standard reaction; c, UV spectra of **20** and product, and HR-MS of the product of the reaction with **20** as the substrate.

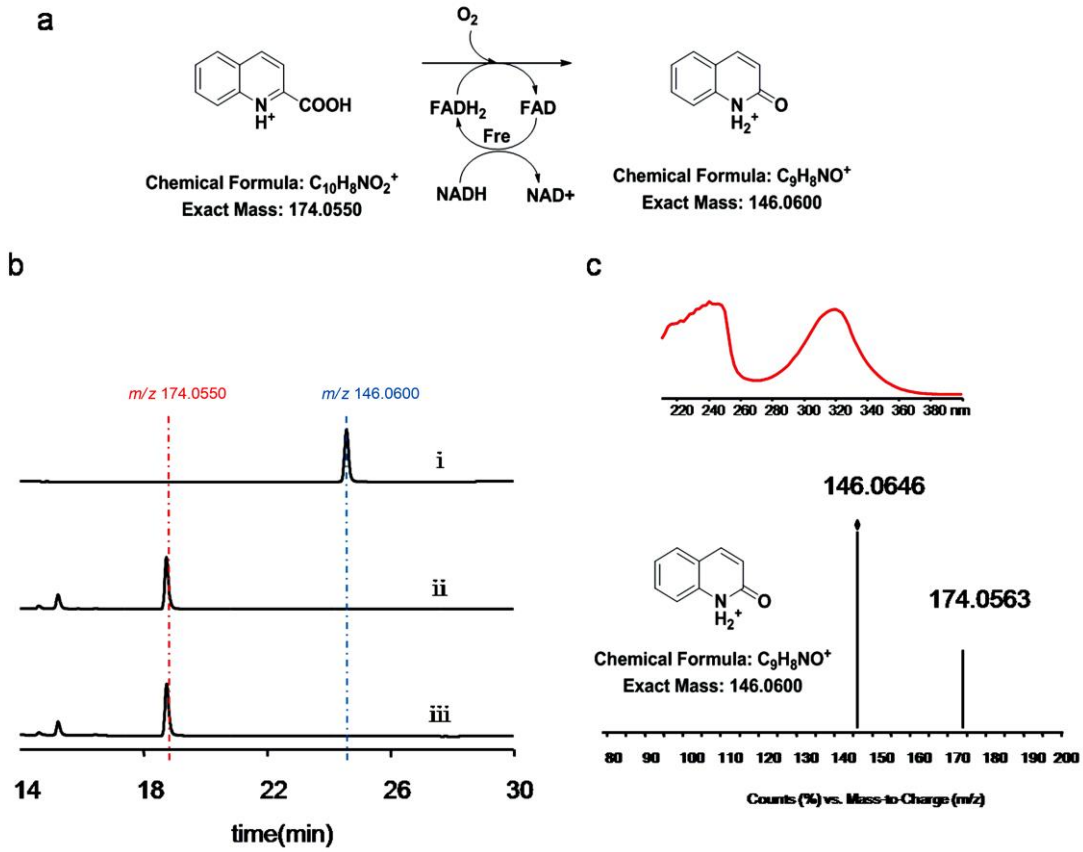

Figure S22. Characterization of the transformation of quinoline-1-carboxylic acid **21** to quinolone catalyzed by FAD. a, Reaction equation; b, The HPLC profiles of the reaction were monitored at 300 nm, (i) authentic quinolone (standard), (ii) control experiment (without FAD), (iii) standard reaction: c, UV spectra of **11** and HR-MS of the product of the reaction with **21** as the substrate.

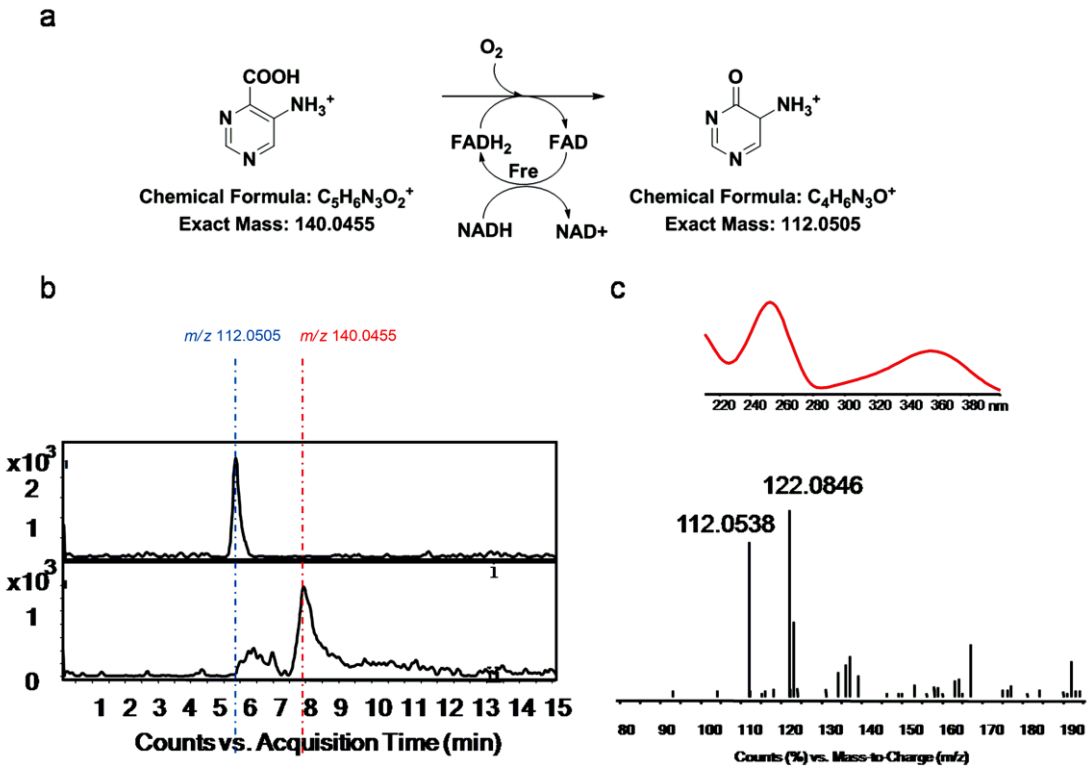

Figure S23. Characterization of the transformation of 5-aminopyrimide-4-carboxylic acid **22** catalyzed by FAD. a, Reaction equation; b, HPLC-MS profiles of the reaction, (i) EIC of MS of the product from **22**, (ii) EIC of MS of **22**; c, UV spectra of **22** and HR-MS of the product from **22** as the substrate.

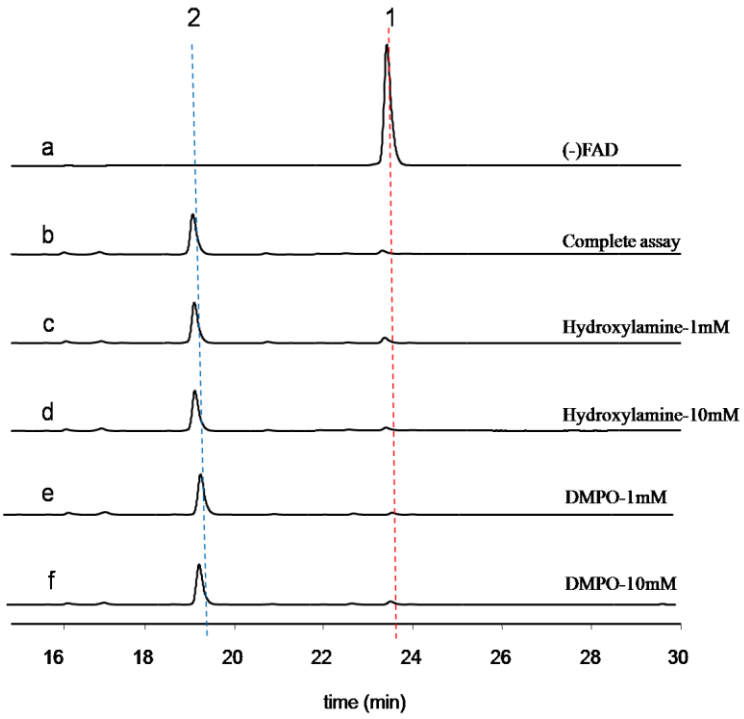

Figure S24. Free radical dropout arrays. (a) Complete assay minus FAD; (b) complete assay; (c) complete assay with 1 mM hydroxylamine; (d) complete assay with 10 mM hydroxylamine; (e) complete assay with 1 mM DMPO; (f) complete assay with 10 mM DMPO. Reactions were carried out under standard condition and analyzed by HPLC with detection at 375 nm.

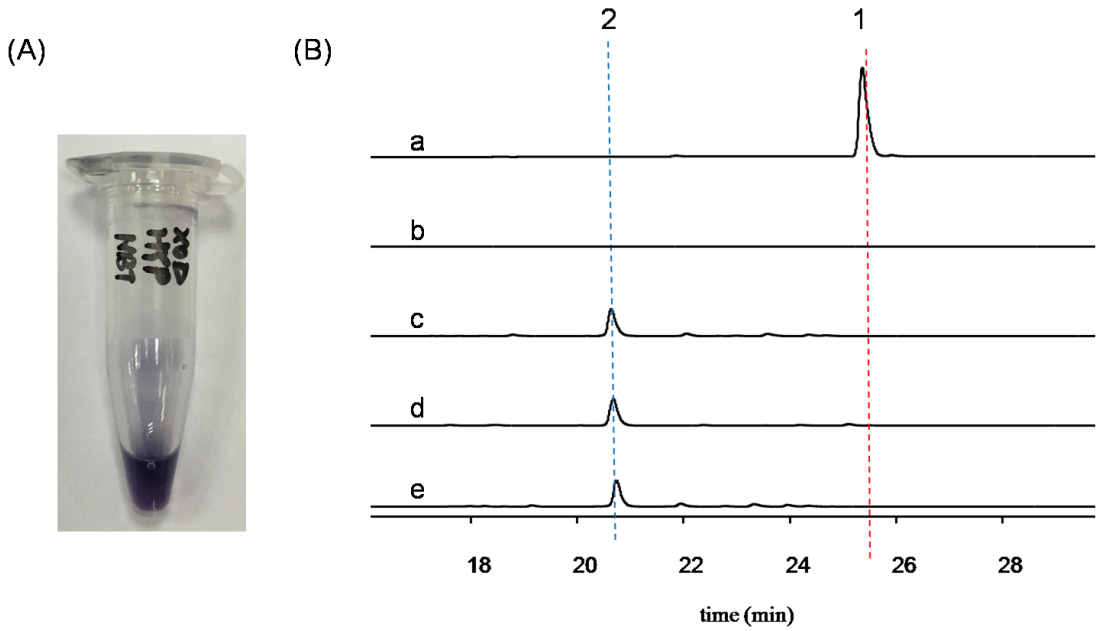

Figure S25 Superoxide dropout assays.

(A) Control experiment: Formazan formation by hypoxanthine/xanthine oxidase system, indicating superoxide formation.

(B) Superoxide dropout assays. (a) streptonigrin incubated in hypoxanthine (200 μM)/xanthine oxidase (0.05 U/mL) system; (b) streptonigrin and potassium superoxide incubated in NaH_2PO_4/Na_2HPO_4 buffer (pH 7.4); (c) standard reaction with SOD (400 U/mL); (d) standard reaction with DMPO (10 mM); (e) The standard reaction