Supporting Information

One-pot chemoenzymatic access to a cefuroxime precursor via C1 extension of furfural

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Materials

Furfural (99%) and methoxyamine hydrochloride (98%) were purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). TEMPO (98%), FHEO (96%) and ThDP (98%) were purchased from Aladdin Biochemical Co., Ltd. (Shanghai, China). FOAc (97%) and SMIA (98%) were purchased from Civic Chemical Technology Co., Ltd. (Shanghai, China). Laccase (from *Trametes* sp., type Y120) is a gift from Amano Enzyme Inc. (Japan). NaOH (96%), NaCl (99.5%), HCl (36-38%), $(NH_4)_2SO_4$ (99%), formaldehyde (40%), $Na_2HPO_4{\cdot}12H_2O$ (99%) and $NaH_2PO_4{\cdot}2H_2O$ (99%) were purchased from Guangshi Regent Technology Co., Ltd. (Guangzhou, China). Kanamycin and isopropyl β-D-thiogalactoside (IPTG) were purchased from Sangon Bioengineering Co., Ltd. (Shanghai, China). Yeast extract and tryptone were from Huankai Biotechnology Co., Ltd. (Guangdong, China). Other chemicals were commercially available. Upon codon optimization, the genes *Sul*PDC linked with MBP[1] and *Pf*BAL (GenBank accession number: AAG02282.1) linked with GB1 were synthesized and cloned to the plasmid pET-28a(+) by GenScript Biotechnology Co., Ltd. (Nanjing, China), furnishing the recombinant plasmids pET28a(+)-MBP-*Sul*PDC and pET28a(+)-GB1-*Pf*BAL, respectively.

Cell cultivation

The plasmids pET28a(+)-MBP-*Sul*PDC/pET28a(+)-GB1-*Pf*BAL were transformed into *E. coli* BL21(DE3), affording the recombinant strains *E. coli*-*Sul*PDC and E. coli-*Pf*BAL, respectively. The strains were pre-cultivated in 30 mL Luria-Bertani (LB) medium containing 50 mg/L kanamycin at 37 °C and 220 rpm overnight. Then, 100 mL LB medium containing 50 mg/L kanamycin was inoculated with 1 mL of overnight culture. Cells were grown at 37 °C and 220 rpm. When the optical density at 600 nm (OD600) reached 0.6-0.8, IPTG was added for inducing enzyme expression at the final concentration of 0.5 mM, followed by incubation at 25 °C and 160 rpm for 20 h. The cells were harvested by centrifugation (8000 rpm, 5 min, 4 °C) and washed twice with 0.85% NaCl solution for the subsequent use.

One-pot chemoenzymatic synthesis of SMIA

To 1 mL of phosphate buffer (50 mM, pH 6) were added 20 mM furfural, 30 mM formaldehyde, 25 g/L *E. coli*-*Sul*PDC cells (wet weight), 0.15 mM ThDP, and 2.5 mM Mg^{2+}, followed by incubation at 35 °C and 150 rpm. After 4 h, the reaction mixture was subjected to

centrifugation to remove cells, and then 3 g/L crude laccase and 10 mM TEMPO were supplemented, followed by incubation at 35 ℃ and 150 rpm. After further reaction of 2 h, approximately 1 mL of methanol and 1.2 equiv. methoxyamine hydrochloride were added to initiate the oximation. The reaction was performed at 4 ℃ with no stirring for 8 h. Aliquots were withdrawn at specified time intervals from the reaction mixtures, and then diluted with the corresponding mobile phase prior to HPLC analysis. All the experiments were conducted at least in duplicate, and all the data were the averages of experimental results. The yields and conversions were determined by HPLC, based on the corresponding calibration curves. The conversion was defined as the percentage of the consumed substrate amount in the initial substrate amount. The yield was defined as the percentage of the measured product amount in the theoretical product amount based on the initial amount of substrate.

Preparative-scale production of FOAc

To 20 mL of phosphate buffer (50 mM, pH 6) were added 20 mM furfural, 1.5 equiv. formaldehyde, 25 g/L *E. coli*-*Sul*PDC cells (wet weight), 0.15 mM ThDP, and 2.5 mM Mg^{2+}, followed by incubation at 35 ℃ and 150 rpm. After 4 h, the reaction mixture was subjected to centrifugation to remove cells, and then 3 g/L crude laccase and 10 mM TEMPO were supplemented, followed by incubation at 35 ℃ and 150 rpm. After further reaction of 4 h, pH of the reaction mixture was tuned to >10 by NaOH, followed by centrifugation. The supernatant was extracted by ethyl acetate (20 mL × 3) to remove TEMPO. The aqueous phase was collected, followed by pH tuning to <1 using H_2SO_4 and adding excess NaCl. The resulting aqueous phase was subjected to extraction by ethyl acetate (20 mL × 3). The organic phases were collected, and dried overnight over anhydrous Na_2SO_4, followed by solvent evaporation at 40 ℃. The target product of 35.8 mg was obtained in brown solid upon drying overnight at 60 ℃.

Preparative-scale production of SMIA

To 50 mL of phosphate buffer (0.2 M, pH 6) were added 100 mM furfural, 1.5 equiv. formaldehyde, 50 g/L *E. coli*-*Sul*PDC cells (wet weight), 0.15 mM ThDP, and 2.5 mM Mg^{2+}, followed by incubation at 35 ℃ and 150 rpm. After 4 h, the reaction mixture was subjected to centrifugation to remove cells, and then 5 g/L crude laccase and 50 mM TEMPO were supplemented, followed by incubation at 35 ℃ and 150 rpm. After further reaction of 20 h, approximately 50 mL of methanol and 1.2 equiv. methoxyamine hydrochloride were added to initiate the oximation. The reaction was performed at 4 ℃ with no stirring. After further reaction of 6 h, most of methanol present in the reaction mixture was evaporated (based on the volume change from approximately 100 mL to 50 mL) under reduced pressure. Then, pH of the resulting reaction mixture was tuned to >10 by NaOH, followed by centrifugation. The supernatant was extracted by ethyl acetate (100 mL × 3) to remove TEMPO. The aqueous phase was collected, followed by pH tuning to <1 using HCl and adding excess NaCl. The resulting aqueous phase was subjected to extraction by ethyl acetate (100 mL × 3). The organic phases were collected, and dried overnight over anhydrous Na_2SO_4, followed by solvent evaporation at 40 ℃. The target product of 0.53 g was obtained in brown oil upon drying overnight at 60 ℃.

Preparative-scale production of MIPA

To 100 mL of 20 vol% DMSO-containing phosphate buffer (0.2 M, pH 6) were added 50 mM benzaldehyde, 1.5 equiv. formaldehyde, 50 g/L *E. coli-Sul*PDC cells (wet weight), 0.15 mM ThDP, and 2.5 mM Mg^{2+}, followed by incubation at 35 °C and 150 rpm. After 10 h, the reaction mixture was subjected to centrifugation to remove cells, and then 5 g/L crude laccase and 25 mM TEMPO were supplemented, followed by incubation at 35 °C and 150 rpm. After further reaction of 30 h, approximately 100 mL of methanol and 1.2 equiv. methoxyamine hydrochloride were added to initiate the oximation. The reaction was performed at 4 °C with no stirring. The reaction was monitored by HPLC (Figure S13). After further reaction of 6 h, most of methanol present in the reaction mixture was evaporated (based on the volume change from approximately 200 mL to 100 mL) under reduced pressure. Then, pH of the resulting reaction mixture was tuned to >10 by NaOH, followed by centrifugation. The supernatant was extracted by ethyl acetate (200 mL × 3) to remove TEMPO. The aqueous phase was collected, followed by pH tuning to <1 using HCl and adding excess NaCl. The resulting aqueous phase was subjected to extraction by ethyl acetate (200 mL × 3). The organic phases were collected. To remove residual DMSO, the organic phase was concentrated to around 100 mL, followed by anti-extraction by pH 1-2 saturated NaCl solution (100 mL × 2). The resultant organic phase was dried overnight over anhydrous Na_2SO_4, followed by solvent evaporation at 40 °C. The solid of 0.61 g was obtained upon drying overnight at 60 °C. HPLC analysis of the obtained product was shown in Figure S14.

HPLC analysis

The HPLC analysis was performed on a Zorbax Eclipse XDB-C18 column (4.6 mm × 250 mm, 5 μm, Agilent, USA) by using a reversed phase HPLC equipped with a Waters 996 photodiode array detector (Waters, USA). The column temperature is 35 °C. The mobile phase was a mixture of acetonitrile/0.4% $(NH_4)_2SO_4$ solution with pH 3.5 (1: 9, v/v) at a flow rate of 0.6 mL/min. The retention times of furfural (maximum absorption wavelength: 276 nm), and FHEO (276 nm), FOAc (282 nm) and SMIA (276 nm) were 13.1, 9.0, 4.5, and 5.3 min, respectively.

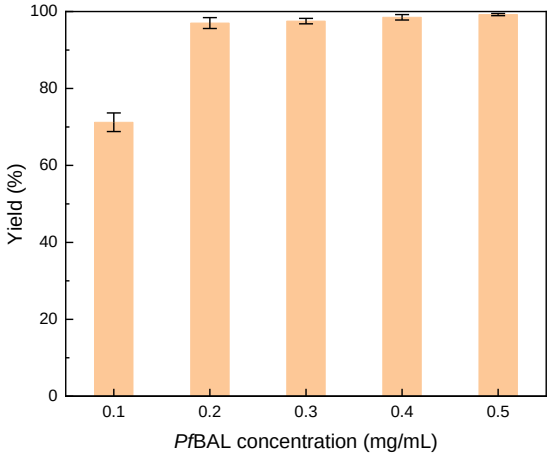


Figure S1. Effect of *Pf*BAL concentrations on FOAc synthesis. Reaction conditions: 10 mM furfural, 3 equiv. HCHO, 0.1-0.5 g/L purified *Pf*BAL, 0.15 mM ThDP, 2.5 mM Mg^{2+}, 1 mL phosphate buffer (50 mM, pH 7), 30°C, 150 rpm, 2 h.

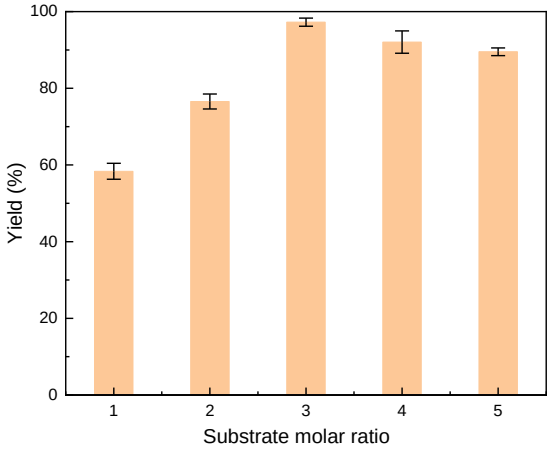


Figure S2. Effect of the molar ratios of HCHO/furfural on FOAc synthesis. Reaction conditions: 10 mM furfural, 1-5 equiv. HCHO, 0.2 g/L purified *Pf*BAL, 0.15 mM ThDP, 2.5 mM Mg^{2+}, 1 mL phosphate buffer (50 mM, pH 7), 30°C, 150 rpm, 1 h.

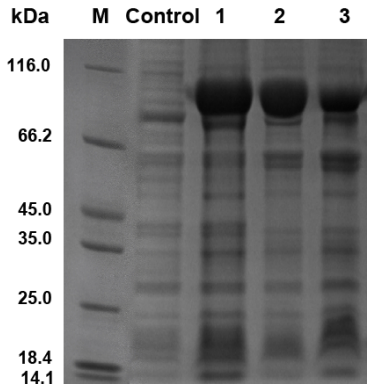


Figure S3. SDS-PAGE of proteins. Lane M: protein marker; Control: all proteins present in *E. coli* cells (harboring empty plasmid) upon ultrasonication; lane 1: all proteins present in *E. coli*-*Sul*PDC cells upon ultrasonication; lane 2: supernatant of *E. coli*-*Sul*PDC upon ultrasonication; lane 3: precipitant of *E. coli*-*Sul*PDC upon ultrasonication.

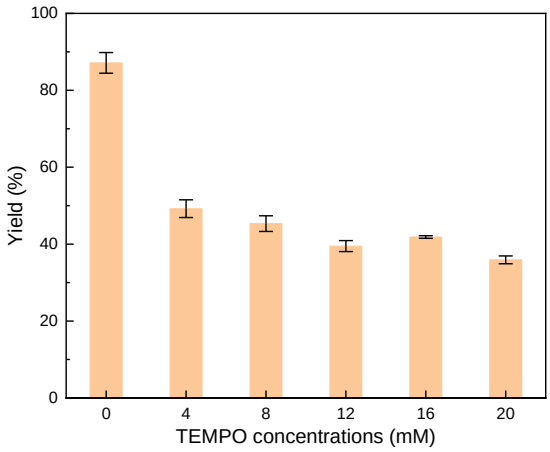


Figure S4. Effect of TEMPO concentrations on whole-cell catalytic FHEO synthesis. Reaction conditions: 40 mM furfural, 1.5 equiv. HCHO, 25 g/L *E. coli-Sul*PDC, 0.15 mM ThDP, 2.5 mM Mg^{2+}, 0-20 mM TEMPO, 1 mL phosphate buffer (50 mM, pH 6), 30℃, 150 rpm, 3h.

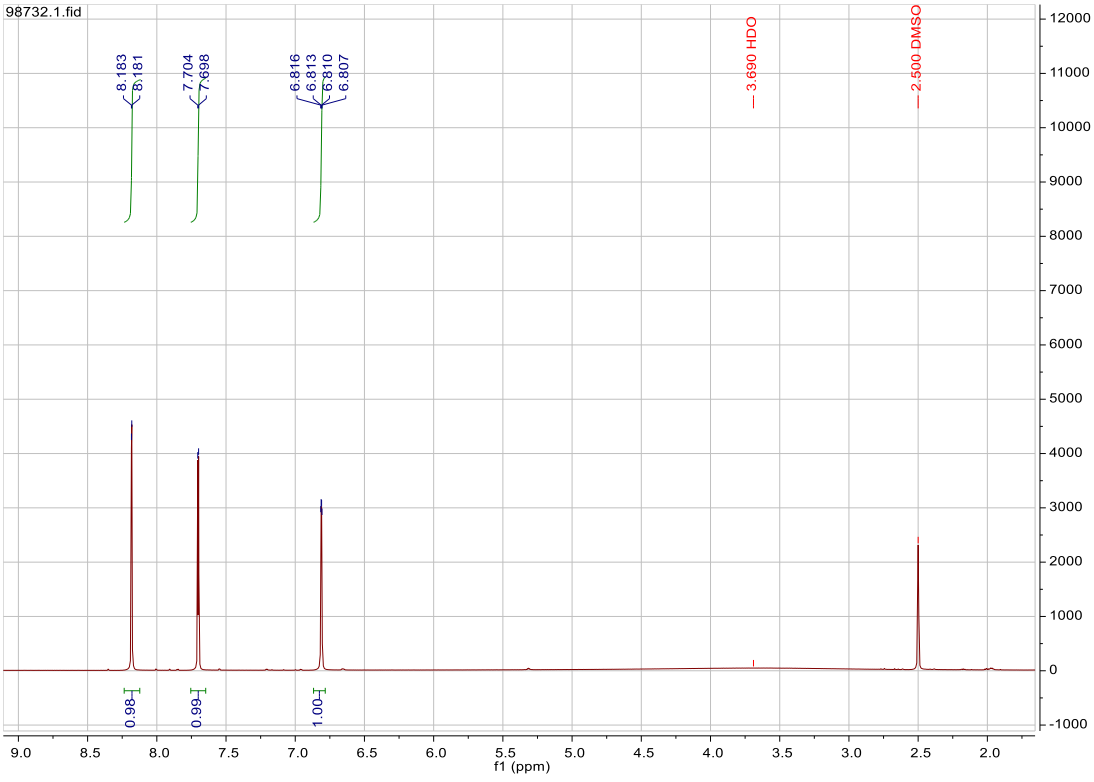


Figure S5. ^{1}H NMR spectrum of FOAc isolated in preparative-scale synthesis (600 MHz, DMSO-d_6).

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.18 (d, J = 1.8 Hz, 1H), 7.70 (d, J = 3.6 Hz), 6.81 (dd, J =1.8 and 3.6 Hz, 1H).

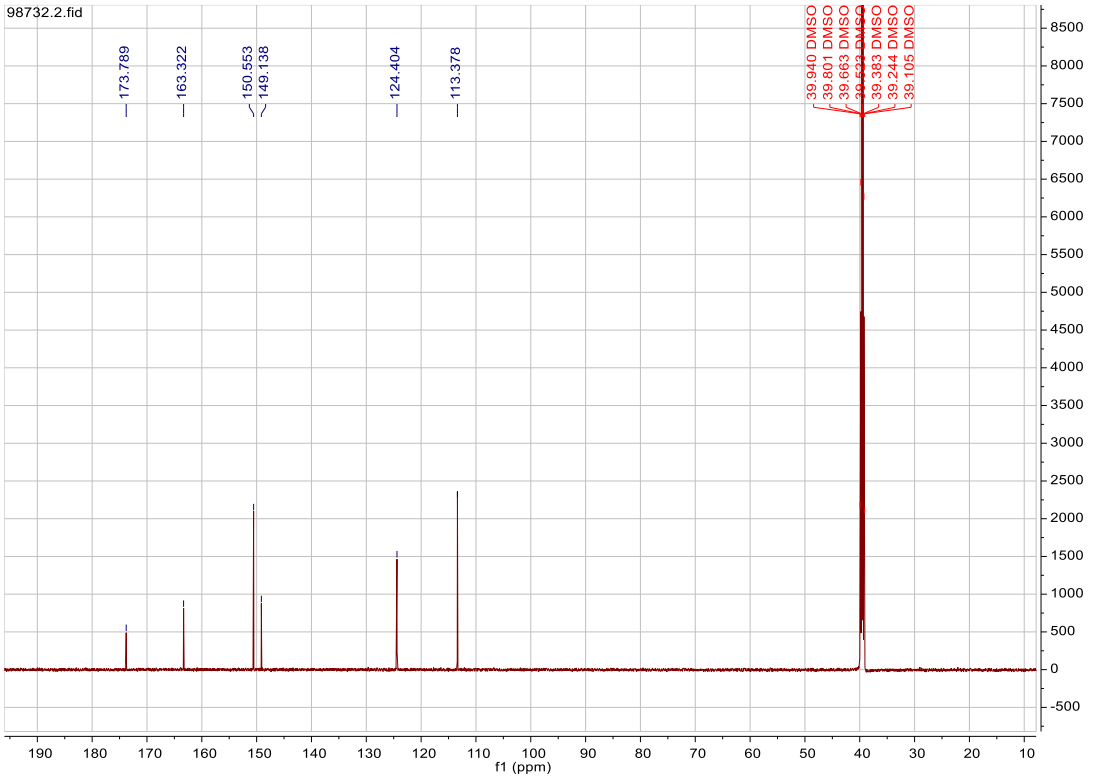
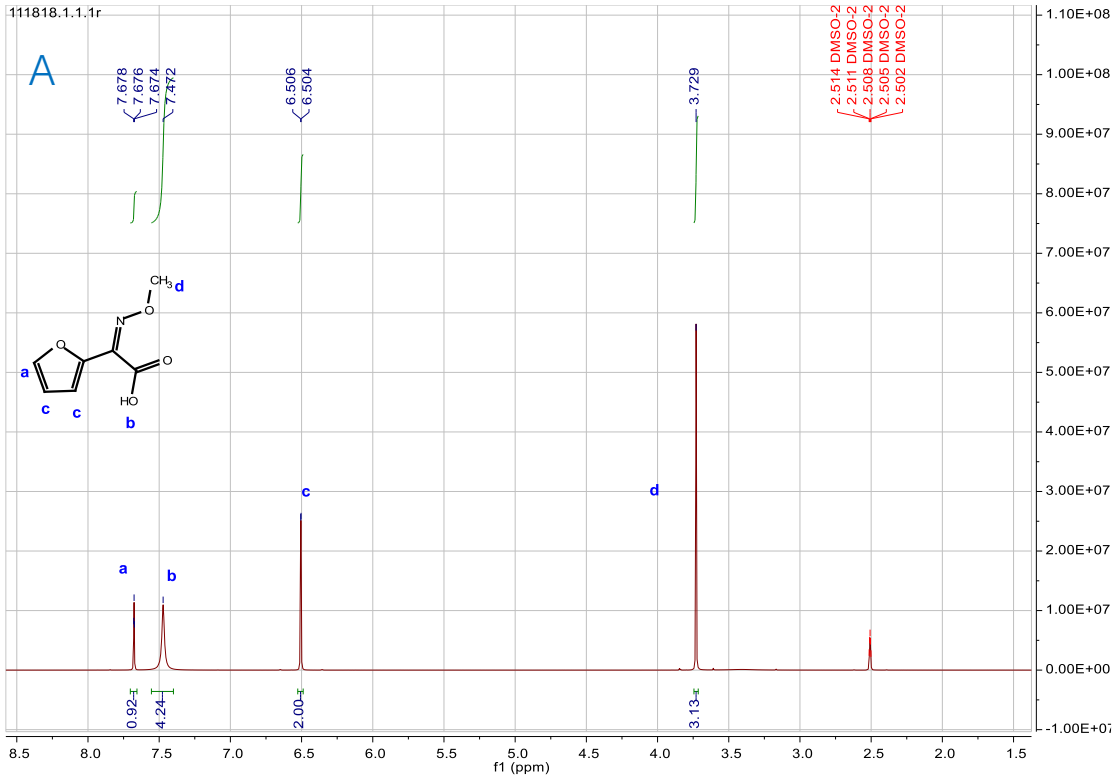


Figure S6. ^{13}C NMR spectrum of FOAc isolated in preparative-scale synthesis (151 MHz, DMSO-d_6).

^{13}C NMR (151 MHz, DMSO-d_6) δ 173.79, 163.32, 150.55, 149.14, 124.40, 113.38.



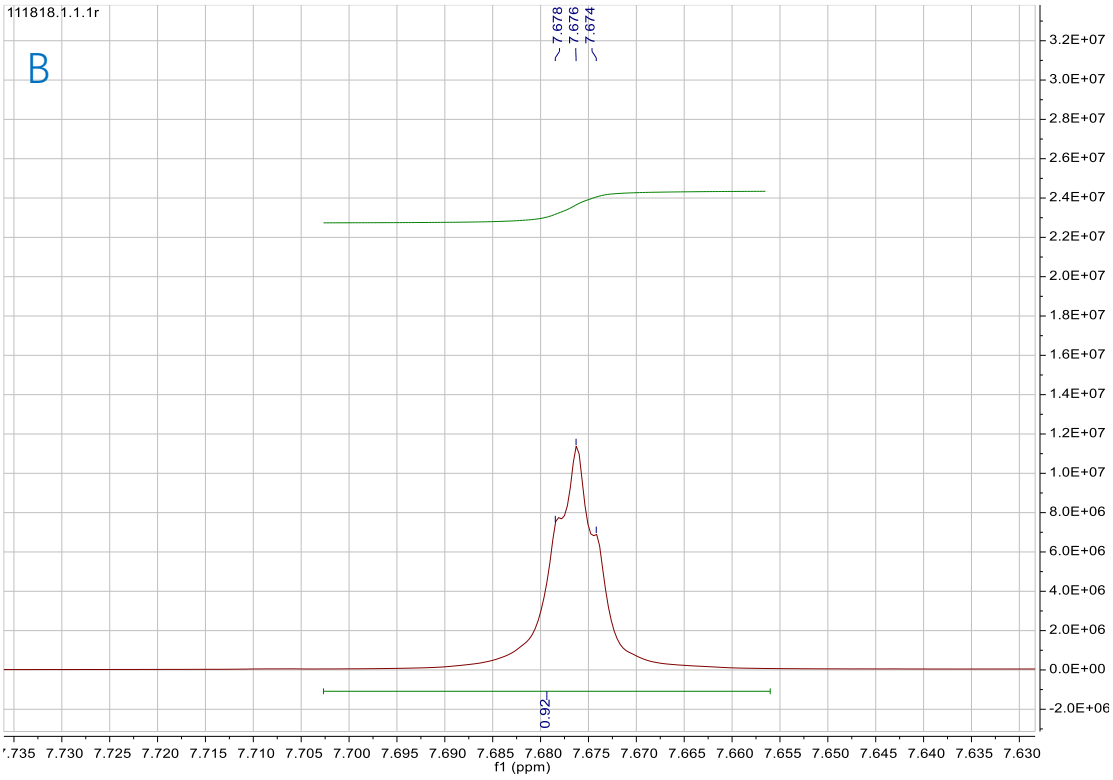


Figure S7. ^{1}H NMR spectrum (A) of SMIA isolated in preparative-scale synthesis (600 MHz, DMSO-d_6) and the details at 7.68 ppm (B).
^{1}H NMR (600 MHz, DMSO-d_6) δ 7.68 (t, 1 H), 7.47 (broad s, 1 H), 6.50 (d, 2H), 3.73 (s, 3H).

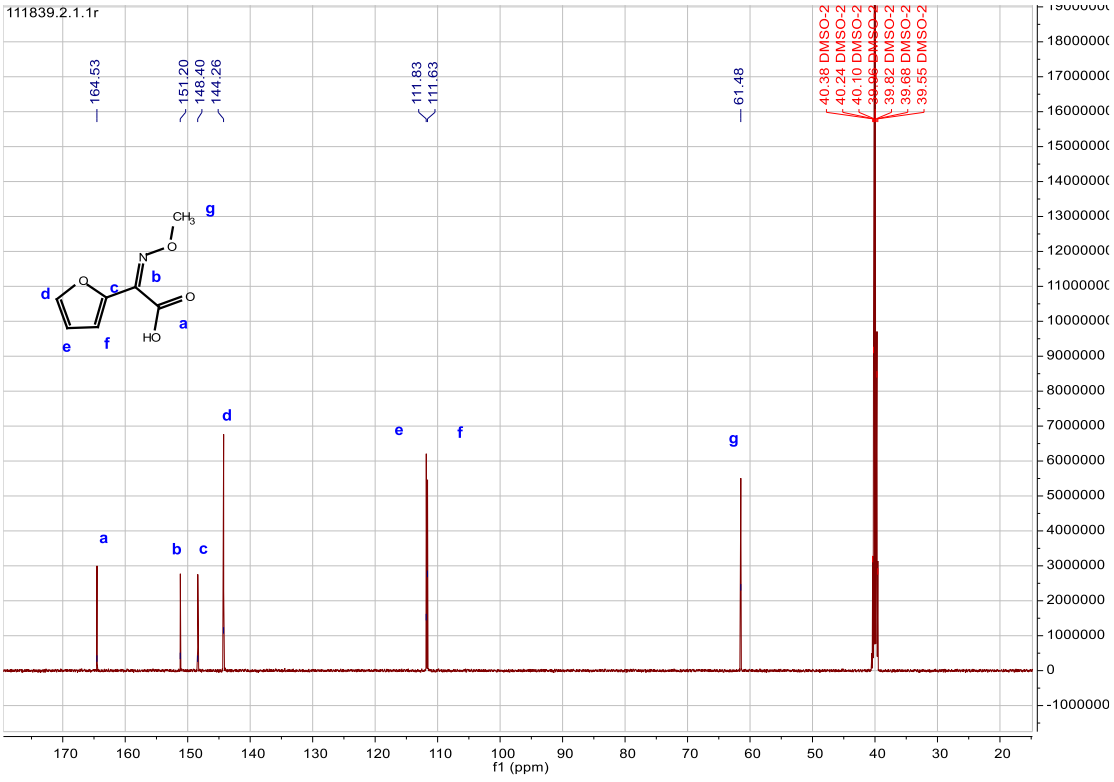


Figure S8. ^{13}C NMR spectrum of SMIA isolated in preparative-scale synthesis (151 MHz, DMSO-d_6).

^{13}C NMR (151 MHz, DMSO-d_6) δ 164.53, 151.20, 148.40, 144.26, 111.83, 111.63, 61.48.

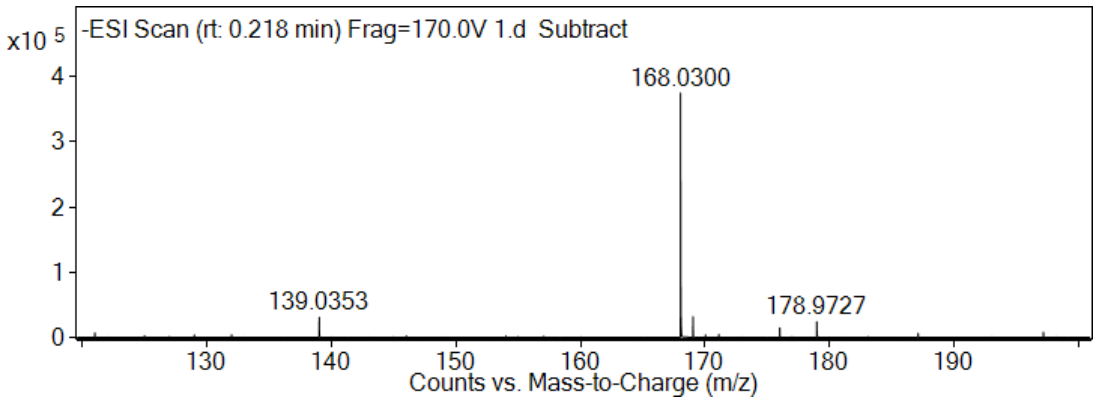


Figure S9. High resolution mass spectrometry (HRMS) of SMIA isolated in preparative-scale synthesis.
HRMS (ESI$^-$): calcd. for C7H6NO4 [M-H]$^-$: 168.0302, found: 168.0300.

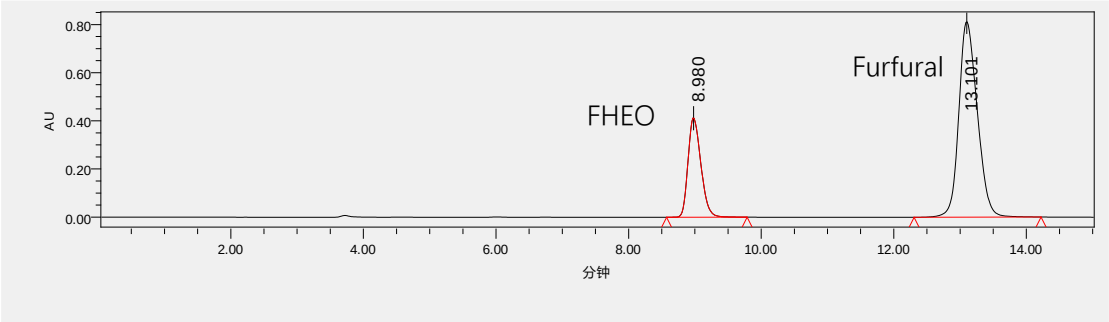


Figure S10. HPLC analysis for the conversion of furfural to FHEO.

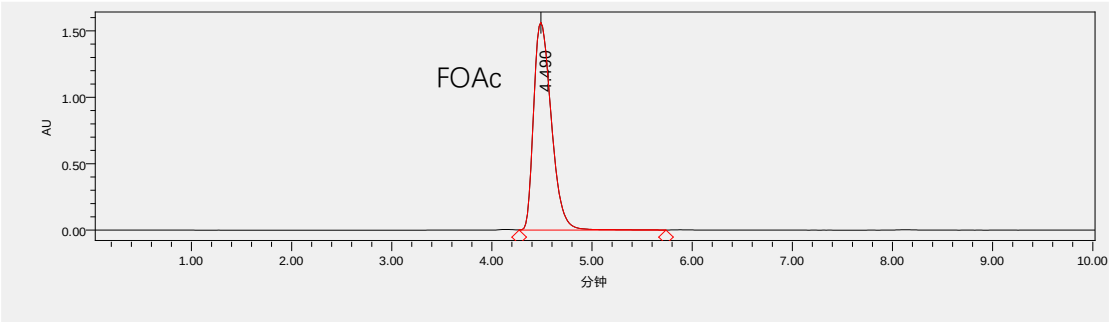


Figure S11. HPLC analysis for the conversion of FHEO to FOAc.

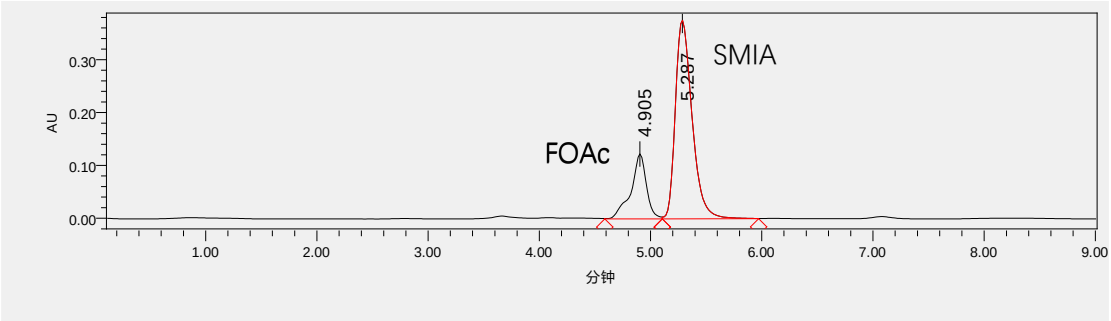


Figure S12. HPLC analysis for the conversion of FOAc to SMIA.

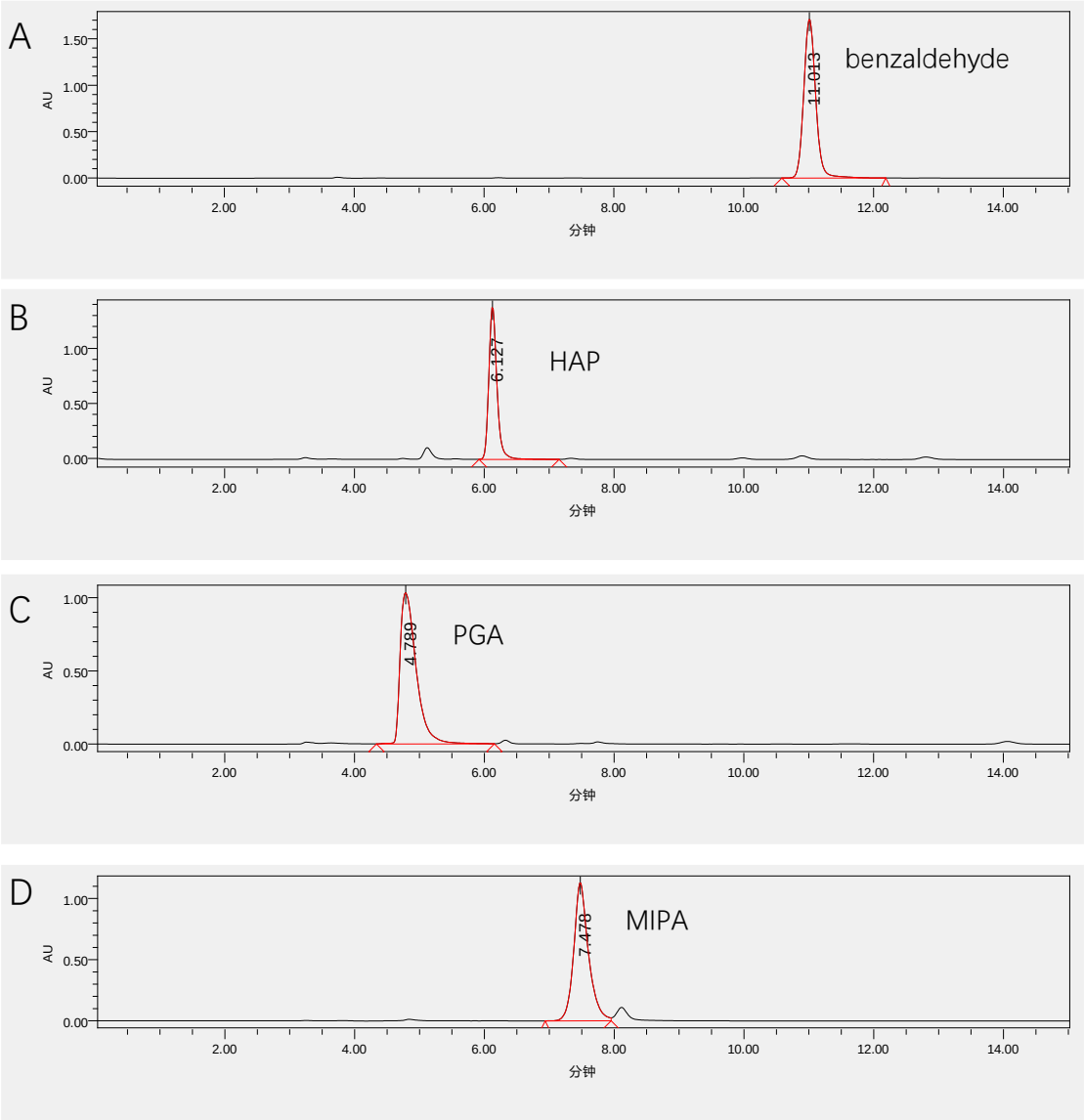


Figure S13. Monitoring the gram-scale conversion of benzaldehyde to MIPA by HPLC: (A) the reaction mixture at the initial stage; (B) the reaction mixture after 10 h; HAP, 2-hydroxyacetophenone (C) the reaction mixture after 40 h; PGA, phenylglyoxylic acid; (D) the reaction mixture after 46 h.

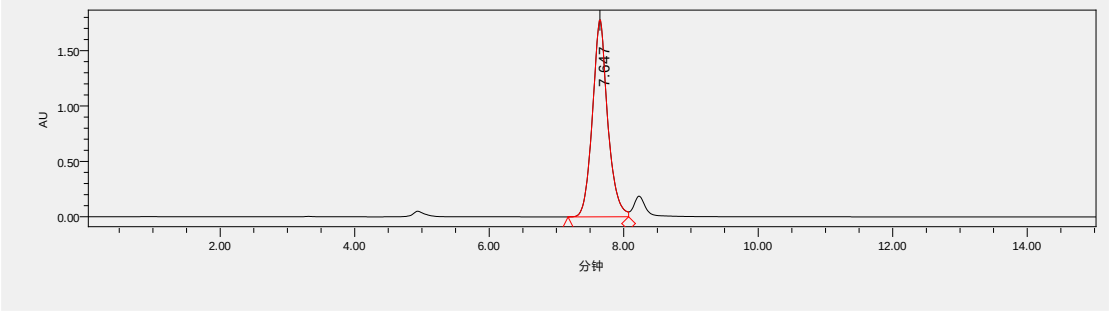


Figure S14. HPLC analysis of the isolated product.

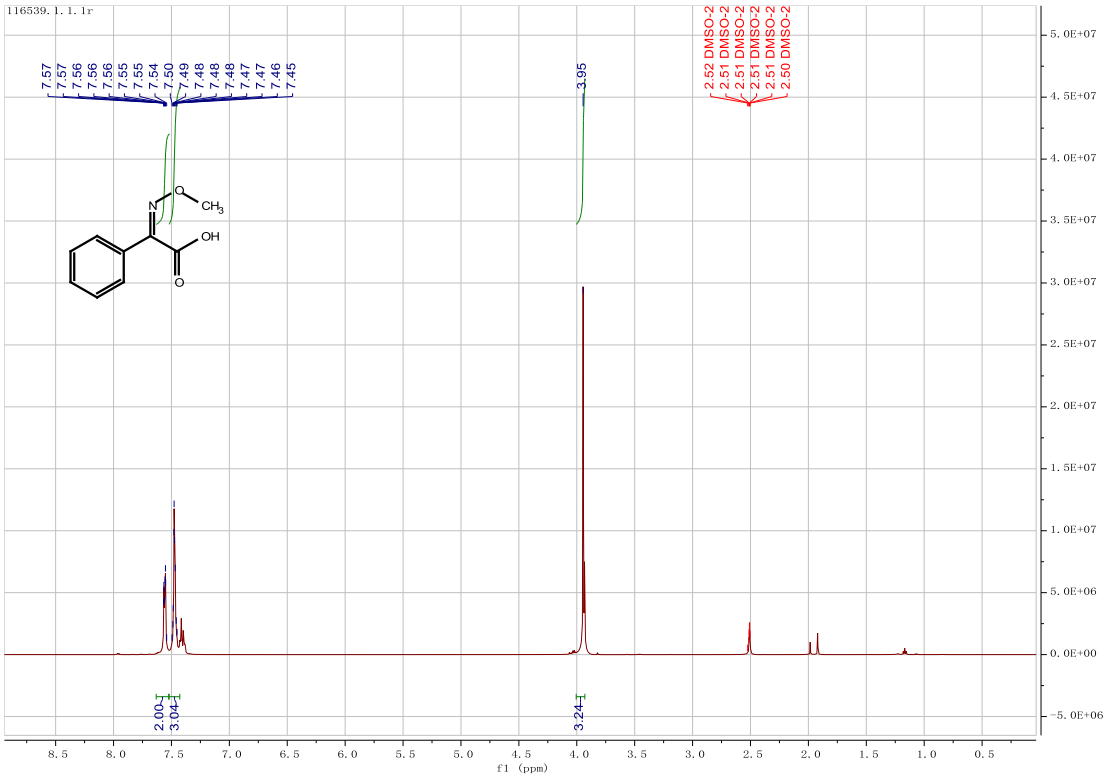


Figure S15. ^{1}H NMR spectrum of MIPA isolated (600 MHz, DMSO-d_6).

^{1}H NMR (600 MHz, DMSO-d_6) δ 7.57-7.54 (m, 2H), 7.50– 7.45 (m, 3H), 3.95 (s, 3H).

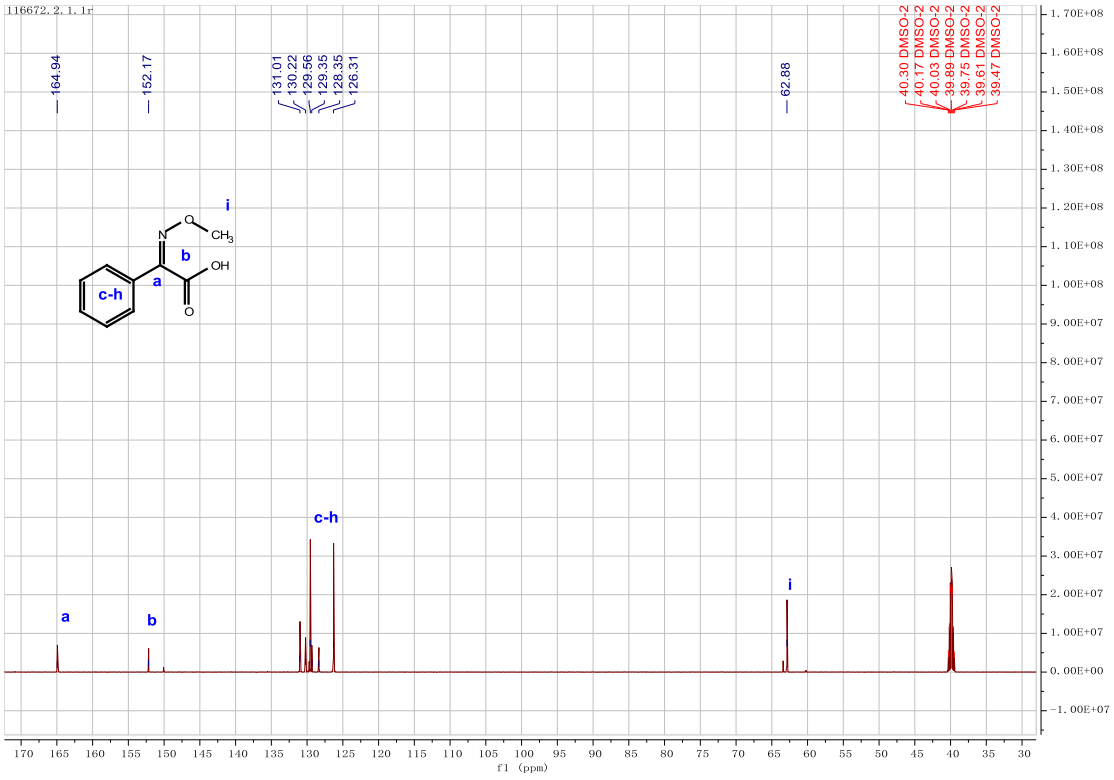


Figure S16. ^{13}C NMR spectrum of MIPA isolated (151 MHz, DMSO-d_6).

^{13}C NMR (151 MHz, DMSO-d_6) δ 164.94, 152.17, 131.01, 130.22, 129.56, 129.35, 128.35,

126.31, 62.88.

References

1. X. Zhang, H. Wei, X. Wei, T. Qi, X. Zong, Z. Liu, J. Qin, X. Gao, G. Zheng and Q. Ma, *Green Chem.*, 2023, **25**, 4713-4722.