Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Constructing a multienzyme cascade redox-neutral system for the synthesis of halogenated indoles

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1. Supplementary Materials and Methods.

1.1. Materials

The chemicals and solvents were commercially available with high purity suitable for analytical applications unless otherwise noted. The gene of all proteins were synthesized by GENEWIZ (Genewiz Biotech Co., Ltd. China).

Table S1. The information of enzymes used in this study.

Enzyme	Source	UniProtKB
HLADH[1]	*Horse liver*	P00327
TADH[1]	*Thermus sp.* ATN1	B2ZRE3
ADH-A[2]	*Rhodococcus ruber* DSM 44541	Q8KLT9
RebH[3]	*Lechevalieria aerocolonigenes*	Q8KHZ8
RebF[3]	*Lechevalieria aerocolonigenes*	Q8KI76
SttH[4]	*Streptomyces toxytricini*	E9P162
BrvH[5]	*Brevundimonas sp.* BAL3	B4WBL8
*Xszen*FHal[6]	*Xenorhabdus szentirmaii* DSM 16338	W1J423

1.2. Experimental Procedures

1.2.1. Expression and purification of enzymes

Flavin-dependent halogenase genes of tryptophan 7-halogenase RebH from *Lechevalieria aerocolonigenes*, tryptophan 5-halogenase *Xszen*FHal from *Xenorhabdus szentirmaii* DSM 16338 and tryptophan 6-halogenase SttH from *Streptomyces toxytricini* were cloned into pET-28a (kanamycin) vector with restriction sites (*NdeI*/*Hind*III) and transformed to *E. coli* BL21 (DE3) pGro7 (chloramphenicol) (Novagene). The gene of brvH from *Brevundimonas sp. BAL3* was cloned into pET-28a vector with restriction sites (*NdeI*/*BamHI*) and further transformed into *E. coli* BL21 (DE3) pGro7 (Novagene).

The genes of flavin reductase RebF from *Lechevalieria aerocolonigenes* and alcohol dehydrogenase from *Thermus sp.* ATN1 (TADH), *Rhodococcus ruber* DSM 44541 (ADH-A) and Horse liver (HLADH) were cloned into pET-28a vector with restriction sites (*NdeI*/*Hind*III) and further transformed into *Escherichia coli* BL21 (DE3).

Expression and purification of halogenases: for primary seed broth cultivation,

the recombinant strains were grown on LB medium (20 mL) supplemented with 50 μg·mL^{-1} kanamycin and 34 μg·mL^{-1} chloramphenicol at 37 °C shaking at 200 rpm for overnight. For enlarged cultivation, 20 mL cell solutions were inoculated to 400 mL LB medium supplemented with 50 μg·mL^{-1} kanamycin and 34 μg·mL^{-1} chloramphenicol at 37 °C shaking at 200 rpm. When the OD_{600} value increased to 0.6-0.8, the cells were induced using 0.1 mM isopropyl-β-dthiogalactopyranoside (IPTG) and 2 mg·mL^{-1} L-arabinose for 20 h at 20 °C. Afterwards, the cell harvested by centrifugation at 4 °C for 10 min at 7104 g and washed with 50 mM KPi buffer (pH 7.0). Thawed the cells and suspended in 20 mL 50 mM KPi buffer (pH 7.0), then lysed with Ultrasonic Cell Disruptor (JY92-II, Scientz Biotech. Co. Ltd.) at 4 °C. Soluble protein was separated from insoluble compounds by centrifugation (10000 × g, 30 min, 4 °C). N-His_6 tag was employed for purification. The elution of the bound enzymes was carried out with imidazole (300 mM imidazole, 50 mM K_2HPO_4, pH 7.0) and resulting enzymes were concentrated and resalted by Millipore (30 kDa). Purified enzymes were stored at -80 °C. The protein concentration was determined by the Bradford method using bovine serum albumin as standard.

Expression and purification of flavin reductase (RebF) and alcohol dehydrogenase (ADH): the recombinant strains were grown on LB medium (20 mL) supplemented with 50 μg·mL^{-1} kanamycin at 37 °C shaking at 200 rpm for overnight. Then 20 mL cell solutions were inoculated to 400 mL LB medium (5% inoculation quantity) supplemented with 50 μg·mL^{-1} kanamycin for the secondary cultivation. When the OD_{600} value increased to 0.6-0.8, the cells were induced overnight using 0.6 mM isopropyl-β-dthiogalactopyranoside (IPTG) at 25 °C. Afterwards, the cell harvested by centrifugation at 4 °C for 15 min at 7104 g and washed with 50 mM KPi buffer (pH 7.0) and stored at -20 °C. Thawed the cells and suspended in 20 mL 50 mM KPi buffer (pH 7.0), then lysed with Ultrasonic Cell Disruptor (JY92-II, Scientz Biotech. Co. Ltd.) at 4 °C. Soluble protein was separated from insoluble compounds by centrifugation (10000 × g, 30 min, 4 °C). N-His_6 tag was employed for purification.

The elution of the bound enzymes was carried out with imidazole (300 mM imidazole, 50 mM K_2HPO_4, pH 7.0) and resulting enzymes were concentrated and resalted by Millipore (30 kDa). Purified enzymes were stored at -80 °C. The protein concentration was determined by the Bradford method using bovine serum albumin as standard.

1.2.2. Reaction methods

ADHs-catalyzed oxidation of 2-(2-aminophenyl)-ethanol: reactions were performed in 2 mL centrifuge tube with 1 mM 2-(2-aminophenyl)-ethanol, various concentration of NAD^+, 2.5 μM HLADH and 50 mM KPi buffer (pH 7.5). The reactions were shaked at 200 rpm at 25 °C, then quenched after 24 h by adding 200 μL of methanol.

RebH/RebF-catalyzed halogenation of indole: Reactions were performed in 2 mL centrifuge tube with 1 mM indole (dissolved in 1% DMSO (v/v)), various concentration of NADH or 0.1 mM NAD^+, 2.5 μM RebH, 10 μM FAD, 1 μM RebF, 2.5 μM HLADH, 650 U/mL Catalase, 40 mM NaCl, various concentration of isopropanol and 50 mM KPi buffer (pH 7.5). The reactions were left shaked at 200 rpm at 25 °C, then quenched after 24 h by adding 200 μL of methanol.

RebH/RebF-catalyzed cascade reactions coupled with HLADH: Cascade reactions were performed in 2 mL centrifuge tube with 1 mM 2-(2-aminophenyl)-ethanol, 0.1 mM NAD^+, 2.5 μM RebH, 10 μM FAD, 1 μM RebF, 2.5 μM HLADH, 650 U/mL Catalase, 40 mM NaCl or NaBr and 50 mM KPi buffer (pH 7.5). The reactions were shaked at 200 rpm at 25 °C, then quenched after 24 h by adding 200 μL of methanol.

RebH-catalyzed cascade reactions with mimic cofactor BNAH: The reactions performed in 2 mL centrifuge tube containing 1 mM 2-(2-aminophenyl)-ethanol, 0.2 mM BNAH (dissolved in 1% DMSO (v/v)), 2.5 μM RebH, 10 μM FAD, 200 μL HLADH lysis solution, 650 U/mL Catalase, 40 mM NaCl and 50 mM KPi buffer (pH 7.5). The reactions were shaked at 200 rpm at 25 °C, then quenched after 12 h by adding 200 μL of methanol.

Scale-up experiment: Cascade reactions were performed in 30 mL KPi buffer (50 mM, pH 7.5) with 10 mM 2-(2-aminophenyl)-ethanol (**1a)**, 2 mM NAD^+, 25 μM Halogenase, 0.1 mM FAD, 50 μM RebF, 15 μM HLADH, 650 U/mL Catalase, 100 mM NaCl. The reactions were shaked at 200 rpm at 25 °C, then quenched after 24 h by adding methanol, **1c** was obtained by preparative chromatography (Biotage SNAP-KP-C18-HS, CH_3CN: H_2O = 1:1) with the quantity of 9.06 mg (isolated yield was 20%).

1.2.3. HPLC methods

Method A: Reactions were monitored using Agilent 1200, LC Column from Agilent Eclipse Plus C18 (250 mm × 4.6 mm × 5 μm), eluent A: H_2O, eluent B: Acetonitrile, flow rate 500 $\mu L \cdot min^{-1}$, eluent B = 70%, λ = 280 nm.

Method B: Reactions were monitored using Agilent 1200, LC Column from Agilent Eclipse Plus C18 (250 mm × 4.6 mm × 5 μm), eluent A: H_2O, eluent B: Acetonitrile, flow rate 500 $\mu L \cdot min^{-1}$, 0-14 min, B = 70%; 14-16 min, B = 70%-60%; 16-20 min, B = 60%; 20-23 min, B = 60%-70%; λ = 280 nm.

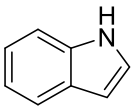
Method C: Reactions were monitored using Waters 2998, LC Column from Agilent Eclipse Plus C18 (250 mm × 4.6 mm × 5 μm), eluent A: Methanol, eluent B: 50 mM Sodium acetate buffer (pH 7.0), eluent B = 50%, 0-17 min, 500 $\mu L \cdot min^{-1}$; 17-20 min, 500-750 $\mu L \cdot min^{-1}$; 20-30 min, 750 $\mu L \cdot min^{-1}$; 30-32 min, 750-500 $\mu L \cdot min^{-1}$; λ = 280 nm.

1.2.4. GC-MS method

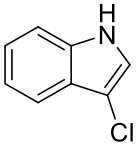
Characterization of products was analyzed by GC-MS instrument (Agilent 7890B GC/5977A MS detector) which was equipped with an HP-5 MS capillary column (30 m × 0.25 mm × 0.25 μm). The injection volume was 2.0 μL with an autosampler and helium was used as a carrier gas with column flow rate of 1.5 $mL \cdot min^{-1}$. The electron ionization (EI) mass spectra in the range of 35-700 (m/z) were recorded in the full scan mode. The detected compounds were identified based on NIST database. The inlet temperature was 250 °C, and the detection temperature of the FID was 300 °C. The column temperature program was an initial temperature of 80 °C, maintained at this

temperature for 3 min, then increased to 200 °C at 20 °C/min, held at this temperature for 2 min and then increased to 235 °C at 15 °C/min, maintained at this temperature for 1 min.

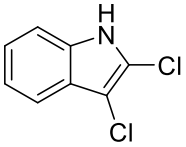
1.2.5 NMR data



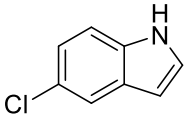
1b (indole**)**: ^{1}H NMR (401 MHz, Methanol-d_4) δ 7.50 (dq, *J* = 7.7, 0.8 Hz, 1H), 7.36-7.31 (m, 1H), 7.17 (d, *J* = 3.1 Hz, 1H), 7.05 (ddd, *J* = 8.1, 6.9, 1.2 Hz, 1H), 6.95 (ddt, *J* = 7.8, 7.0, 0.7 Hz, 1H), 6.39 (dd, *J* = 3.1, 0.9 Hz, 1H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 136.3, 128.3, 124.1, 121.3, 119.8, 118.6, 110.7, 100.9.



1c (3-chloroindole): ^{1}H NMR (401 MHz, Methanol-d_4) δ 7.49-7.45 (m, 1H), 7.34 (dt, *J* = 8.2, 0.9 Hz, 1H), 7.19 (s, 1H), 7.13 (ddd, *J* = 8.2, 7.0, 1.3 Hz, 1H), 7.06 (ddd, *J* = 8.0, 7.0, 1.0 Hz, 1H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 135.4, 125.1, 122.1, 121.2, 119.4, 117.1, 111.4, 104.5.

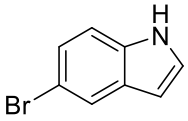


1d (2,3-dichloroindole): 1H NMR (401 MHz, Methanol-d_4) δ 7.43-7.38 (m, 1H), 7.27 (dt, J = 8.2, 0.9 Hz, 1H), 7.15 (ddd, J = 8.2, 7.2, 1.2 Hz, 1H), 7.09 (ddd, J = 8.0, 7.1, 1.1 Hz, 1H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 133.8, 125.1, 122.6, 120.2, 116.7, 110.9, 101.4.

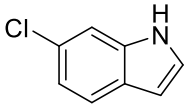


1e (5-chloroindole): ^{1}H NMR (401 MHz, Methanol-d_4) δ 7.48 (dd, *J* = 2.0, 0.5 Hz, 1H), 7.30 (dd, *J* = 8.6, 0.7 Hz, 1H), 7.25-7.21 (m, 1H), 7.01 (dd, *J* = 8.6, 2.0 Hz,

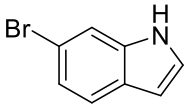
1H), 6.37 (dd, *J* = 3.1, 0.9 Hz, 1H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 134.7, 129.1, 125.8, 124.3, 120.9, 119.1, 111.9, 100.5.



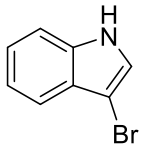
1f (5-bromoindole): ^{1}H NMR (401 MHz, Methanol-d_4) δ 7.64 (dt, *J* = 1.8, 0.7 Hz, 1H), 7.29-7.20 (m, 2H), 7.14 (dt, *J* = 8.6, 1.4 Hz, 1H), 6.37 (dt, *J* = 3.2, 0.9 Hz, 1H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 125.7, 123.6, 122.2 (d, *J* = 2.8 Hz), 112.4, 100.7.



1g (6-chloroindole): ^{1}H NMR (401 MHz, Methanol-d_4) δ 7.45 (dd, *J* = 8.5, 0.6 Hz, 1H), 7.34 (dt, *J* = 1.6, 0.8 Hz, 1H), 7.20 (d, *J* = 3.2 Hz, 1H), 6.94 (dd, *J* = 8.5, 1.9 Hz, 1H), 6.40 (dd, *J* = 3.2, 0.9 Hz, 1H).^{13}C NMR (101 MHz, Methanol-d_4) δ 136.7, 126.7 (d, *J* = 11.0 Hz), 125.2, 120.8, 119.2, 110.6, 101.2.



1h (6-bromoindole): ^{1}H NMR (401 MHz, Methanol-d_4) δ 7.50 (dd, *J* = 1.8, 0.9 Hz, 1H), 7.41 (d, *J* = 8.4 Hz, 1H), 7.19 (dd, *J* = 3.2, 0.8 Hz, 1H), 7.07 (dt, *J* = 8.5, 1.2 Hz, 1H), 6.40 (dd, *J* = 3.1, 1.0 Hz, 1H).^{13}C NMR (101 MHz, Methanol-d_4) δ 137.2, 127.1, 125.1, 121.8, 121.2, 114.2, 113.6, 101.2.



1i (3-bromoindole): ^{1}H NMR (401 MHz, Methanol-d_4) δ 7.41 (dt, *J* = 7.8, 1.1 Hz, 1H), 7.35 (dt, *J* = 8.2, 1.0 Hz, 1H), 7.24 (s, 1H), 7.13 (ddd, *J* = 8.2, 7.0, 1.3 Hz, 1H), 7.07 (ddd, *J* = 8.0, 7.0, 1.1 Hz, 1H).^{13}C NMR (101 MHz, Methanol-d_4) δ 135.8, 126.7, 123.7, 122.1, 119.6, 118.0, 111.4, 89.5.

2. Supplementary Figures

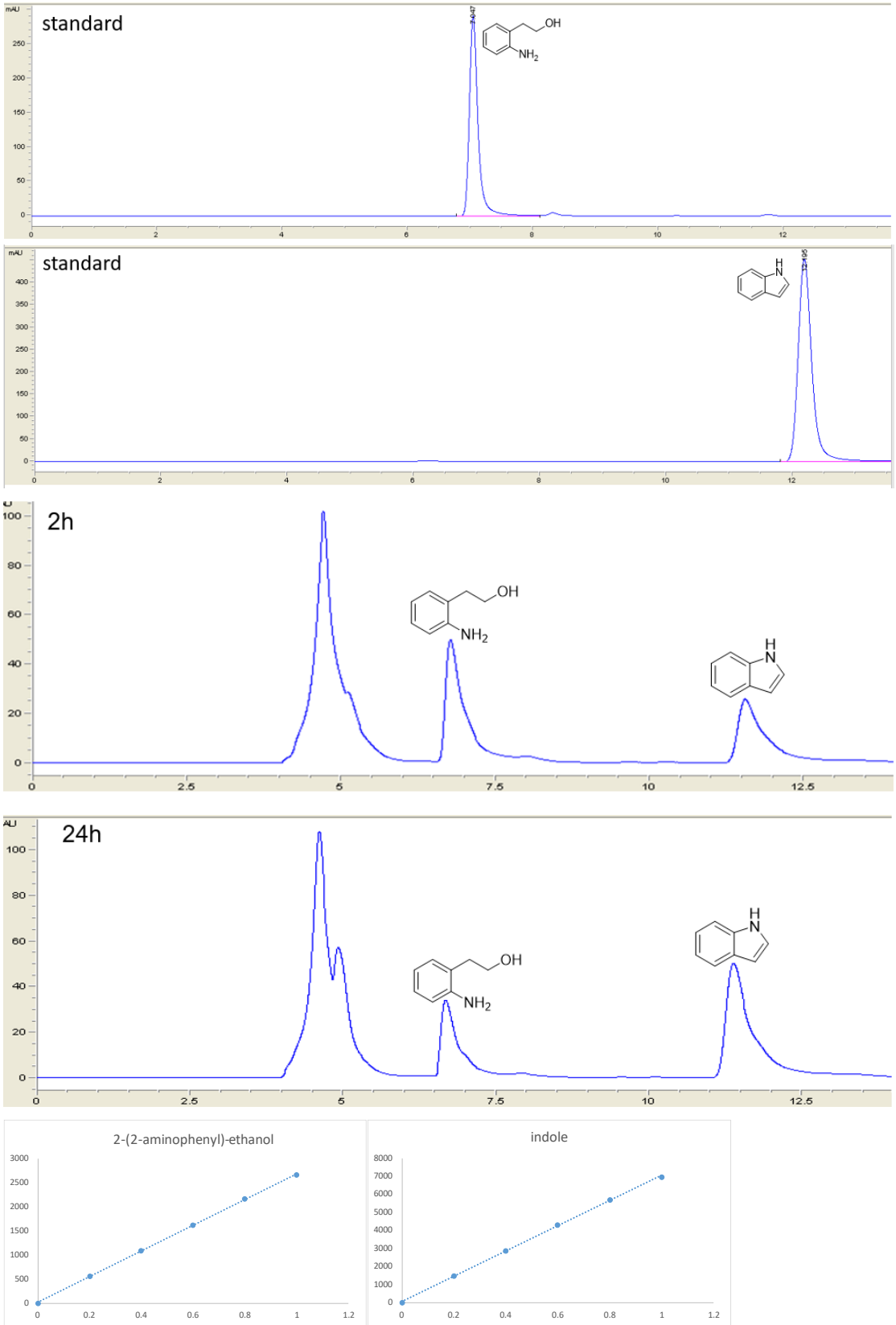


Fig. S1 HLADH catalyzed 2-(2-aminophenyl)-ethanol to indole. (HPLC method A). 1 mM 2-(2-aminophenyl)-ethanol, 0.1 mM NAD^+, 2.5 μM HLADH and 50 mM KPi buffer (pH 7.5).

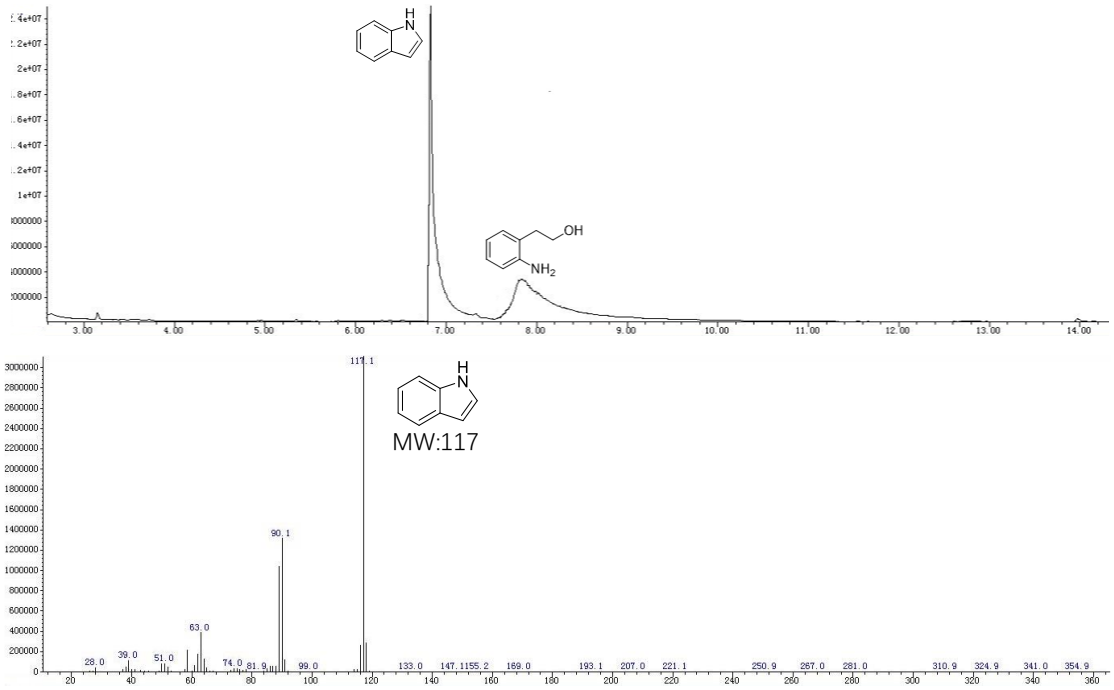
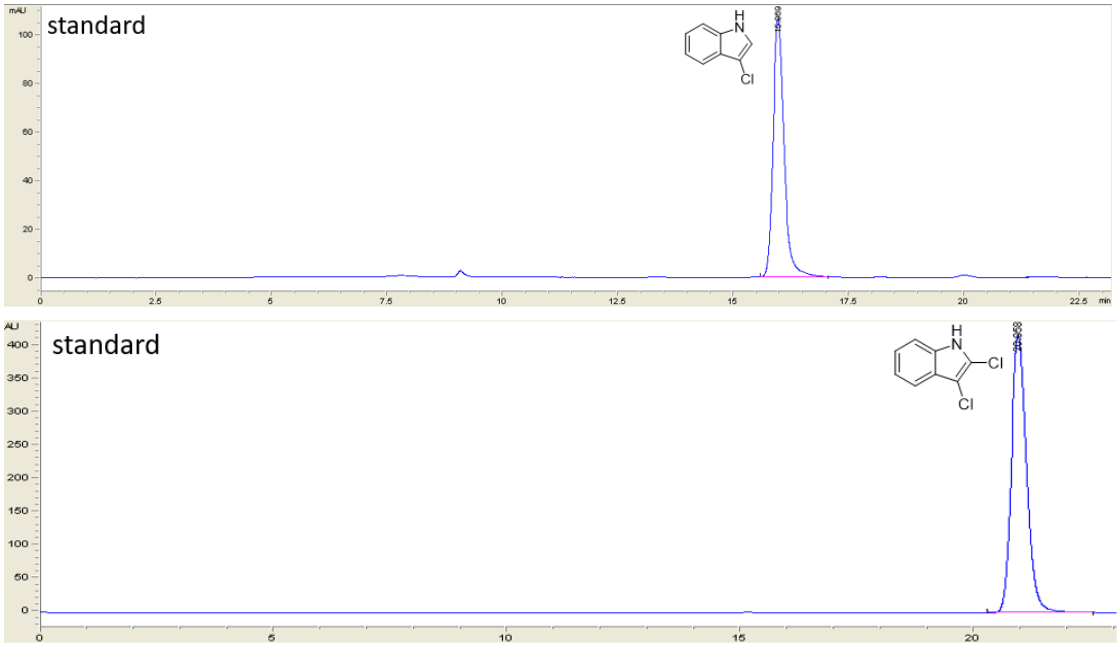


Fig. S2 HLADH catalyzed 2-(2-aminophenyl)-ethanol to indole. (GC-MS). 1 mM 2- (2-aminophenyl)-ethanol, 0.1 mM NAD^+, 2.5 μM HLADH and 50 mM KPi buffer (pH 7.5). The reactions were shaked at 200 rpm at 25 °C



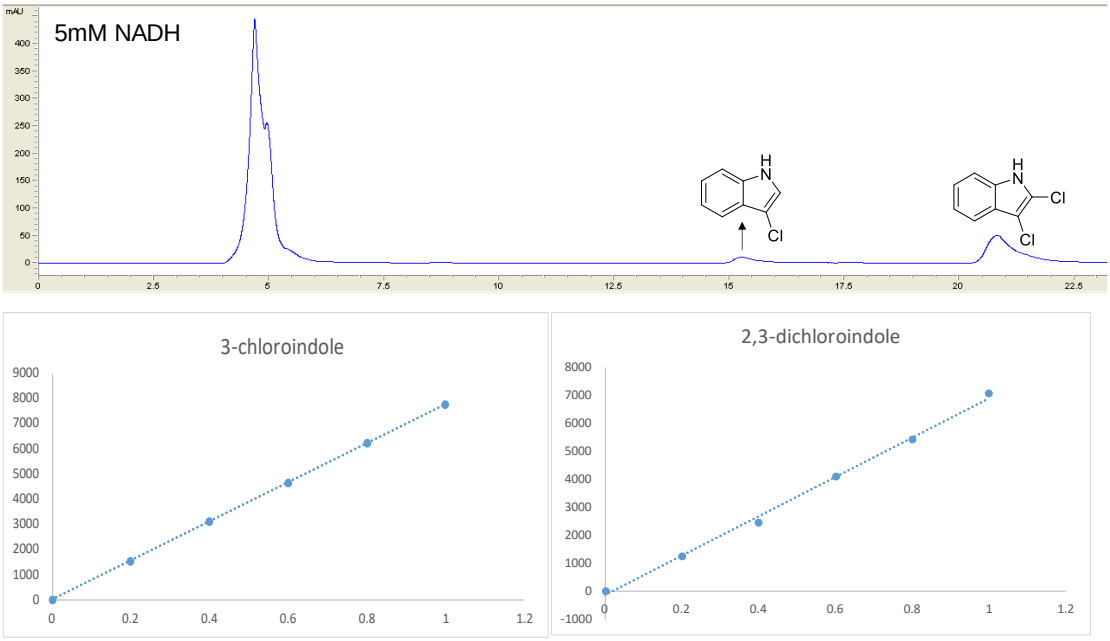


Fig. S3 RebH/RebF-catalyzed halogenation of indole by adding 5 mM NADH. (HPLC method B). 1 mM 2-(2-aminophenyl)-ethanol, 5 mM NADH, 2.5 μM HLADH and 50 mM KPi buffer (pH 7.5). 25 °C, 24 h.

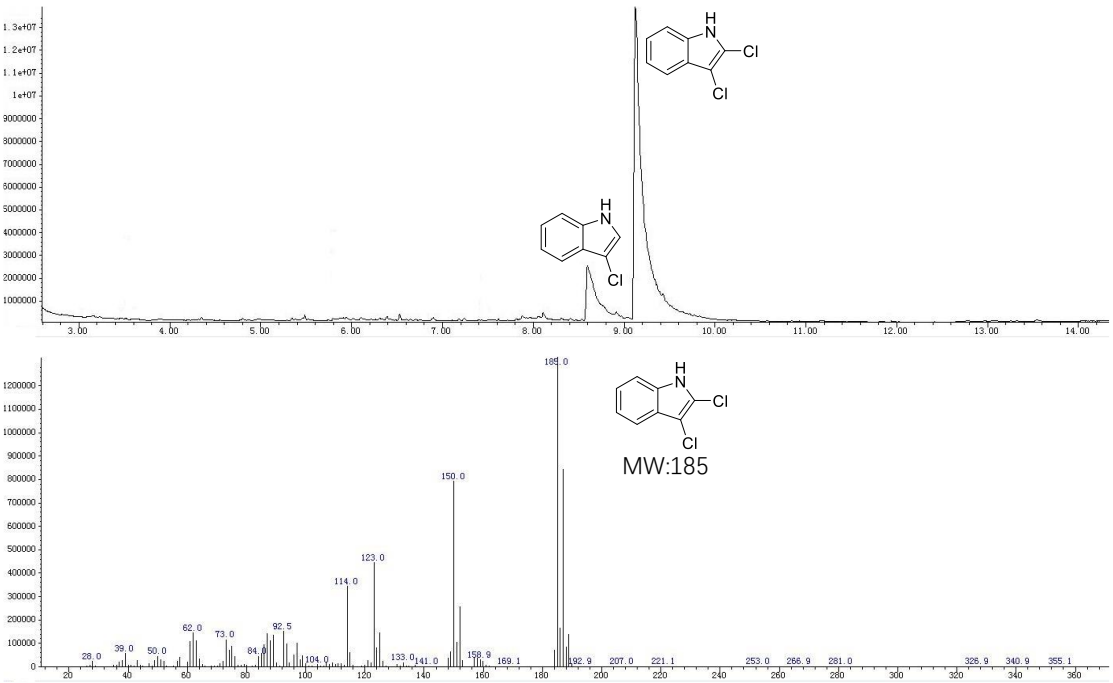


Fig. S4 RebH/RebF-catalyzed halogenation of indole by adding 5 mM NADH. (GC-MS). 1 mM 2-(2-aminophenyl)-ethanol, 5 mM NADH, 2.5 μM HLADH and 50 mM KPi buffer (pH 7.5). 25 °C, 24 h.

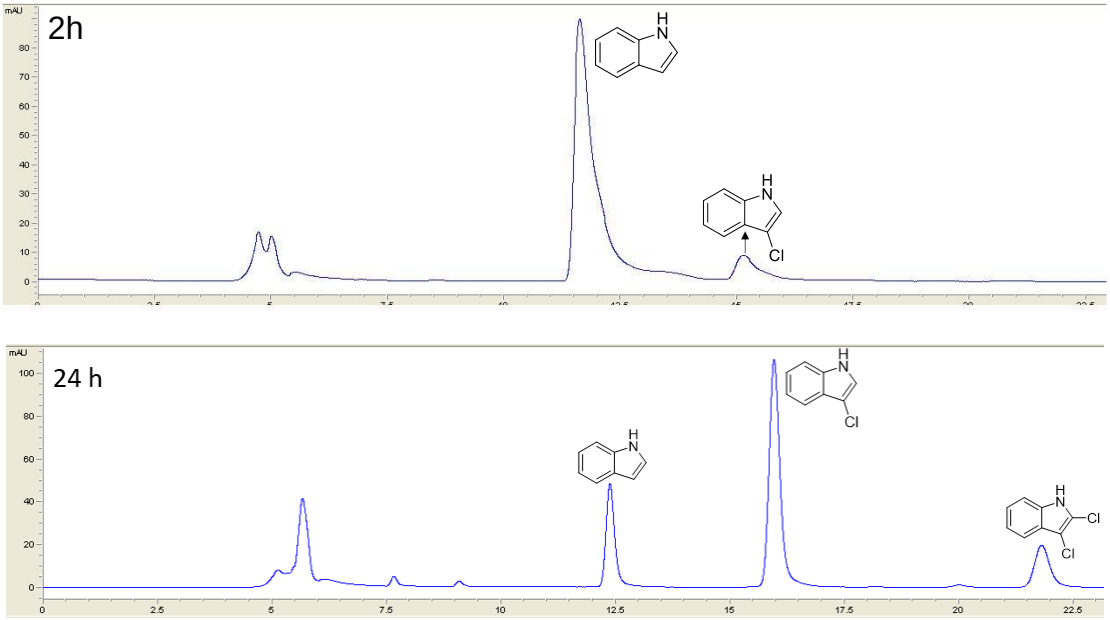


Fig. S5 RebH/RebF-catalyzed halogenation of indole coupled with HLADH by adding 50 mM isopropanol. (HPLC method B). 1 mM indole (dissolved in 1% DMSO (v/v)), 0.1 mM NAD^+, 2.5 µM RebH, 10 µM FAD, 1 µM RebF, 2.5 µM HLADH, 650 U/mL Catalase, 40 mM NaCl, 50 mM isopropanol and 50 mM KPi buffer (pH 7.5), 25 °C, 24 h.

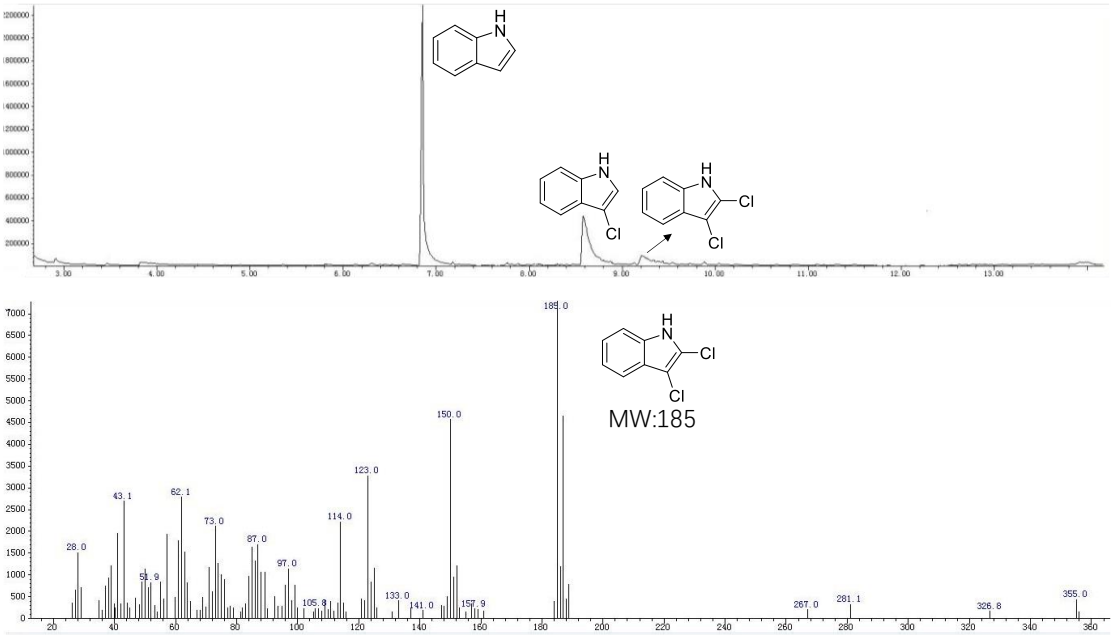


Fig. S6 RebH/RebF-catalyzed halogenation of indole coupled with HLADH by adding 50 mM isopropanol. (GC-MS). 1 mM indole (dissolved in 1% DMSO (v/v)), 0.1 mM NAD^+, 2.5 µM RebH, 10 µM FAD, 1 µM RebF, 2.5 µM HLADH, 650 U/mL Catalase, 40 mM NaCl, 50 mM isopropanol and 50 mM KPi buffer (pH 7.5), 25 °C, 24 h.

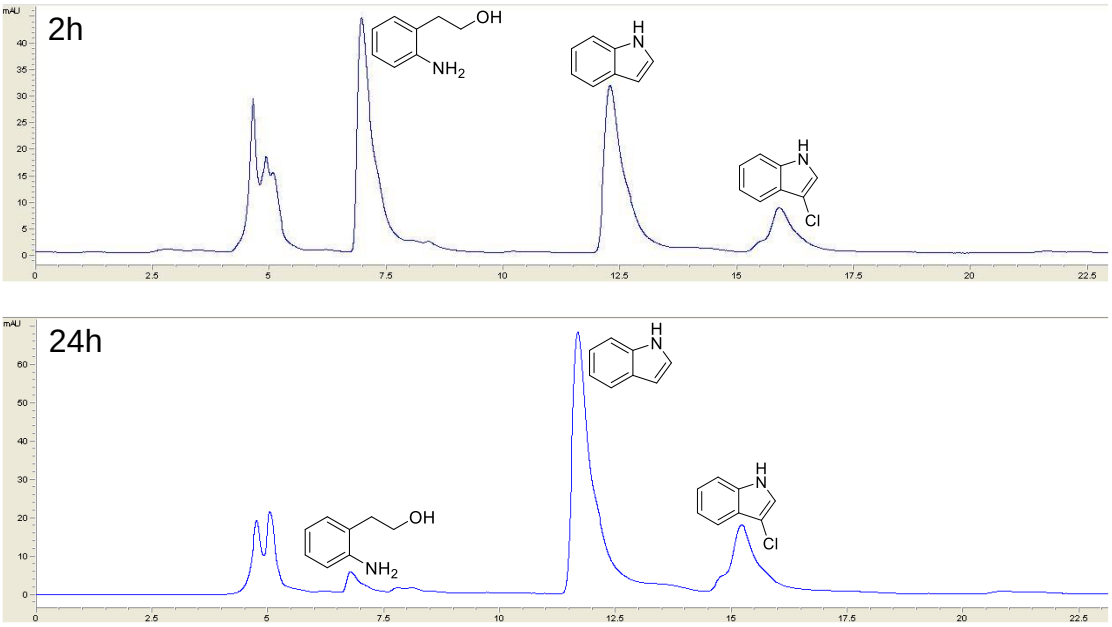


Fig. S7 Biosynthesis of 3-Cl-indole from 2-(2-aminophenyl)-ethanol through the multienzyme redox-neutral cascade system. (HPLC method B). 1 mM 2-(2-aminophenyl)-ethanol, 0.1 mM NAD^+, 2.5 μM RebH, 10 μM FAD, 1 μM RebF, 2.5 μM HLADH, 650 U/mL Catalase, 40 mM NaCl, 50 mM KPi buffer (pH 7.5), 25 °C, 24 h.

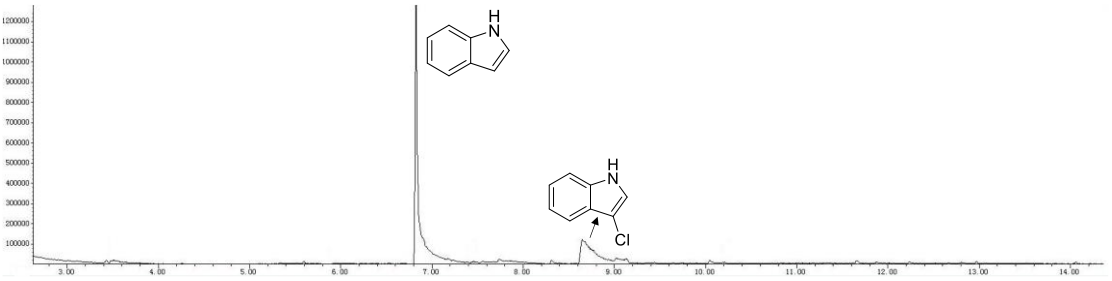


Fig. S8 Biosynthesis of 3-Cl-indole from 2-(2-aminophenyl)-ethanol through the multienzyme redox-neutral cascade system. (GC-MS). 1 mM 2-(2-aminophenyl)-ethanol, 0.1 mM NAD^+, 2.5 μM RebH, 10 μM FAD, 1 μM RebF, 2.5 μM HLADH, 650 U/mL Catalase, 40 mM NaCl, 50 mM KPi buffer (pH 7.5), 25 °C, 24 h.

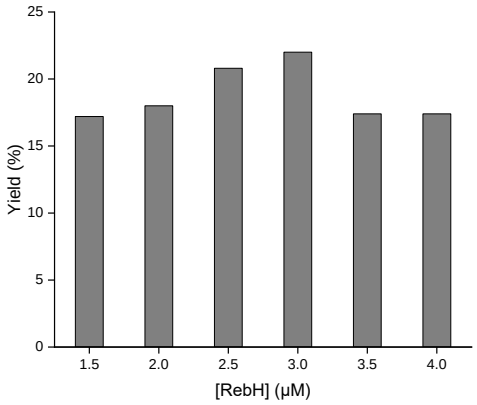


Fig. S9 Effect of different concentration of RebH on the reaction. 2.5 μM HLADH, 1 mM 2-(2-aminophenyl)-ethanol, 0.1 mM NAD^+, 10 μM FAD, 1 μM RebF, 40 mM NaCl, 650 U/mL Catalase, 50 mM KPi buffer (pH 7.5), 25 °C, 24 h. Average values for duplicate reactions are displayed.

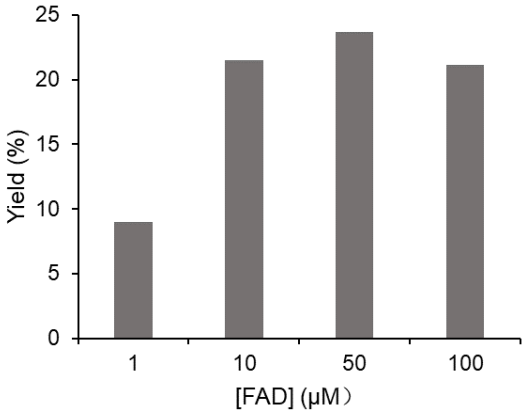
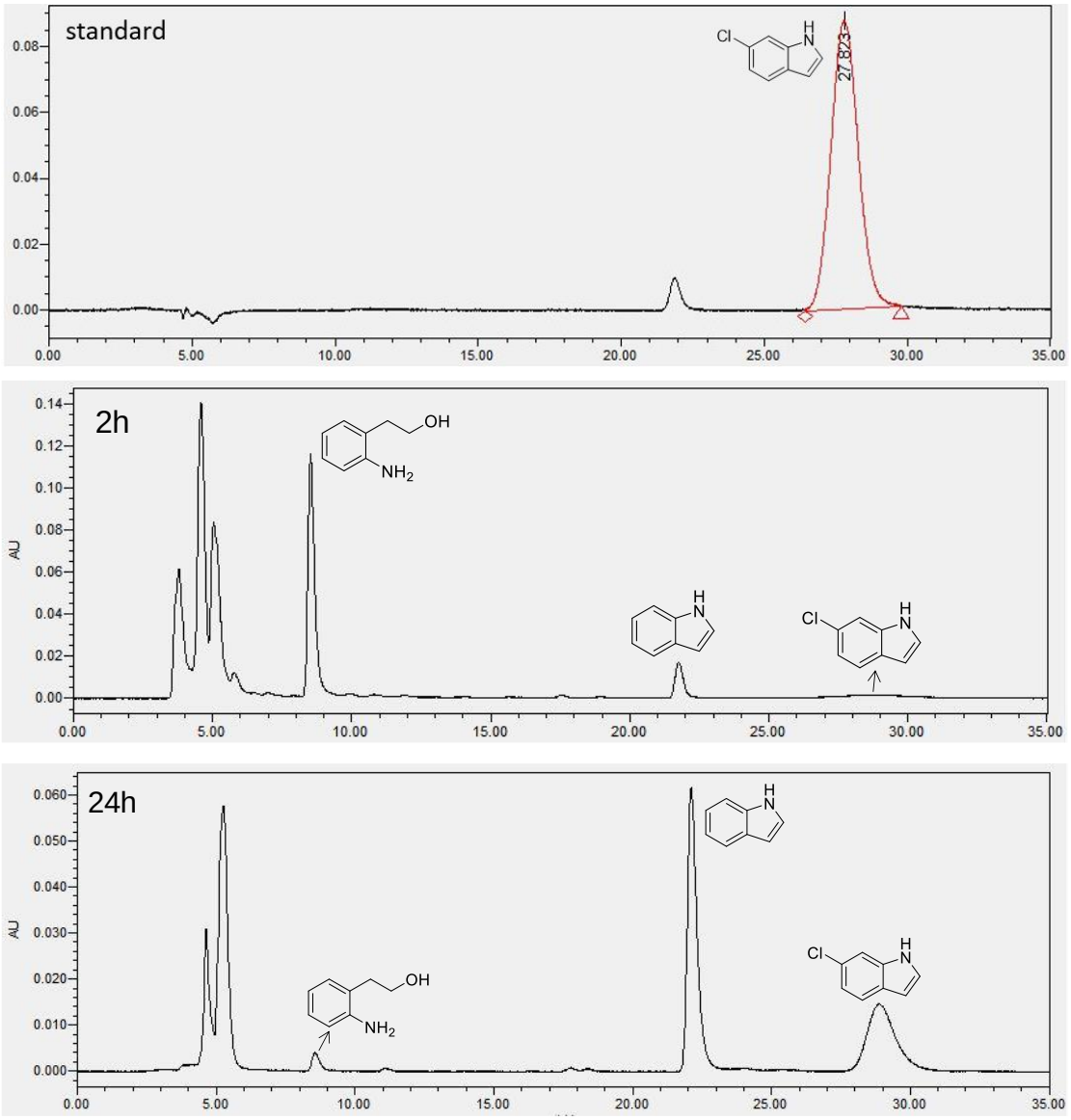


Fig. S10 Effect of different concentration of FAD on the reaction. 2.5 μM HLADH, 1 mM 2-(2-aminophenyl)-ethanol, 0.1 mM NAD^+, 1 μM RebF, 2.5 μM RebH, 40 mM NaCl, 650 U/mL Catalase, 50 mM KPi buffer (pH 7.5), 25 °C, 24 h. Average values for duplicate reactions are displayed.



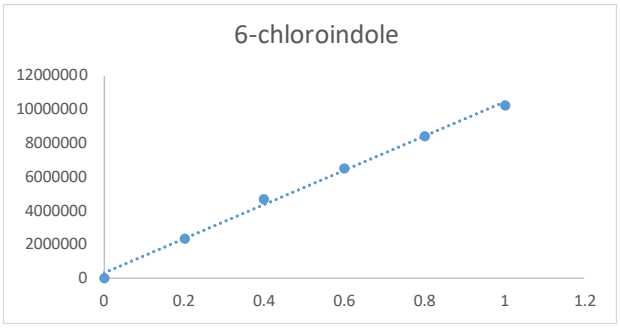


Fig. S11 Biosynthesis of 6-Cl-indole via SttH/RebF coupled with HLADH. (HPLC method C). 1 mM 2-(2-aminophenyl)-ethanol, 0.1 mM NAD^+, 2.5 μM SttH, 10 μM FAD, 10 μM RebF, 2.5 μM HLADH, 650 U/mL Catalase, 40 mM NaCl, 50 mM KPi buffer (pH 7.5), 25 °C, 24 h.

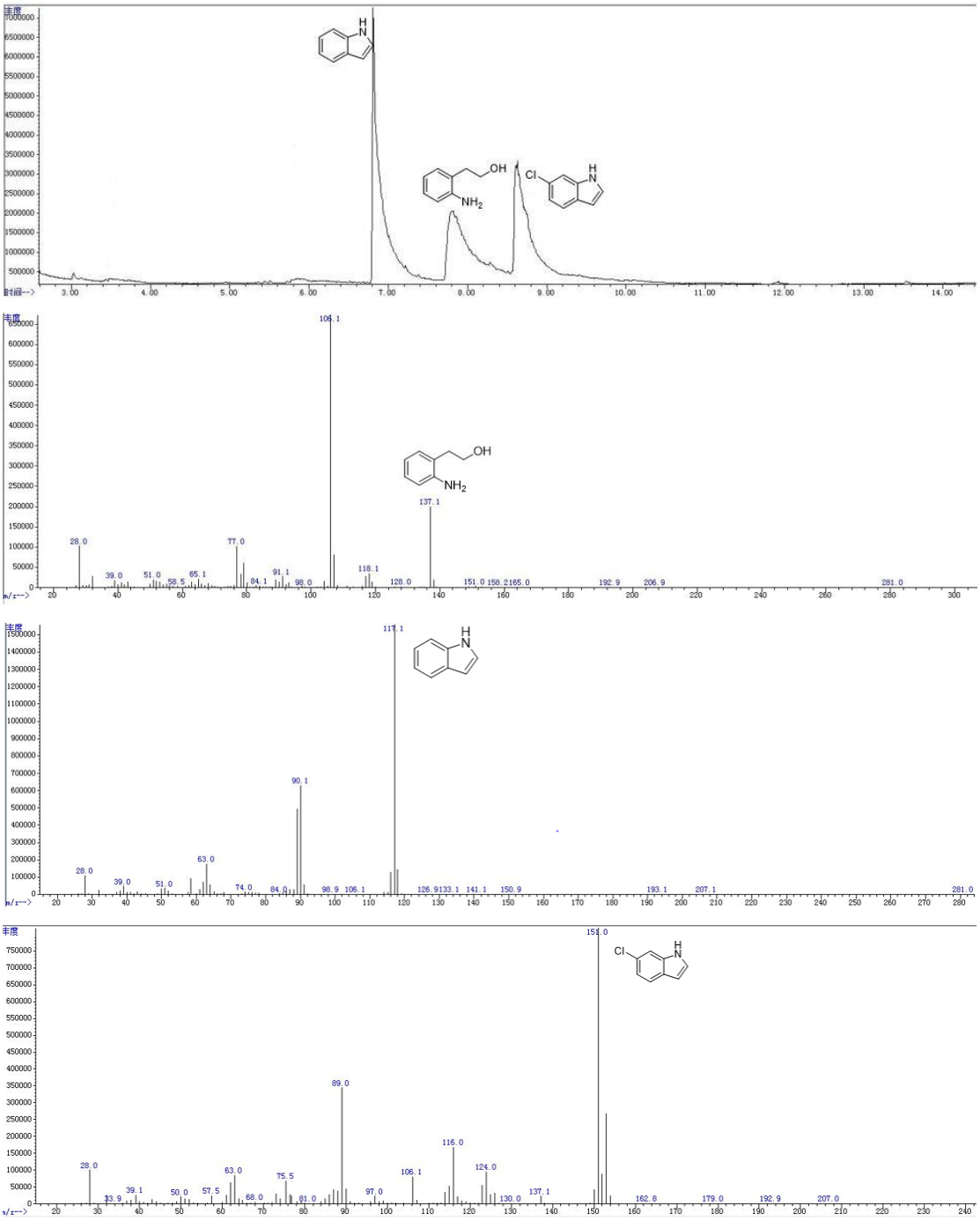


Fig. S12 Biosynthesis of 6-Cl-indole via SttH/RebF coupled with HLADH. (GC-MS).

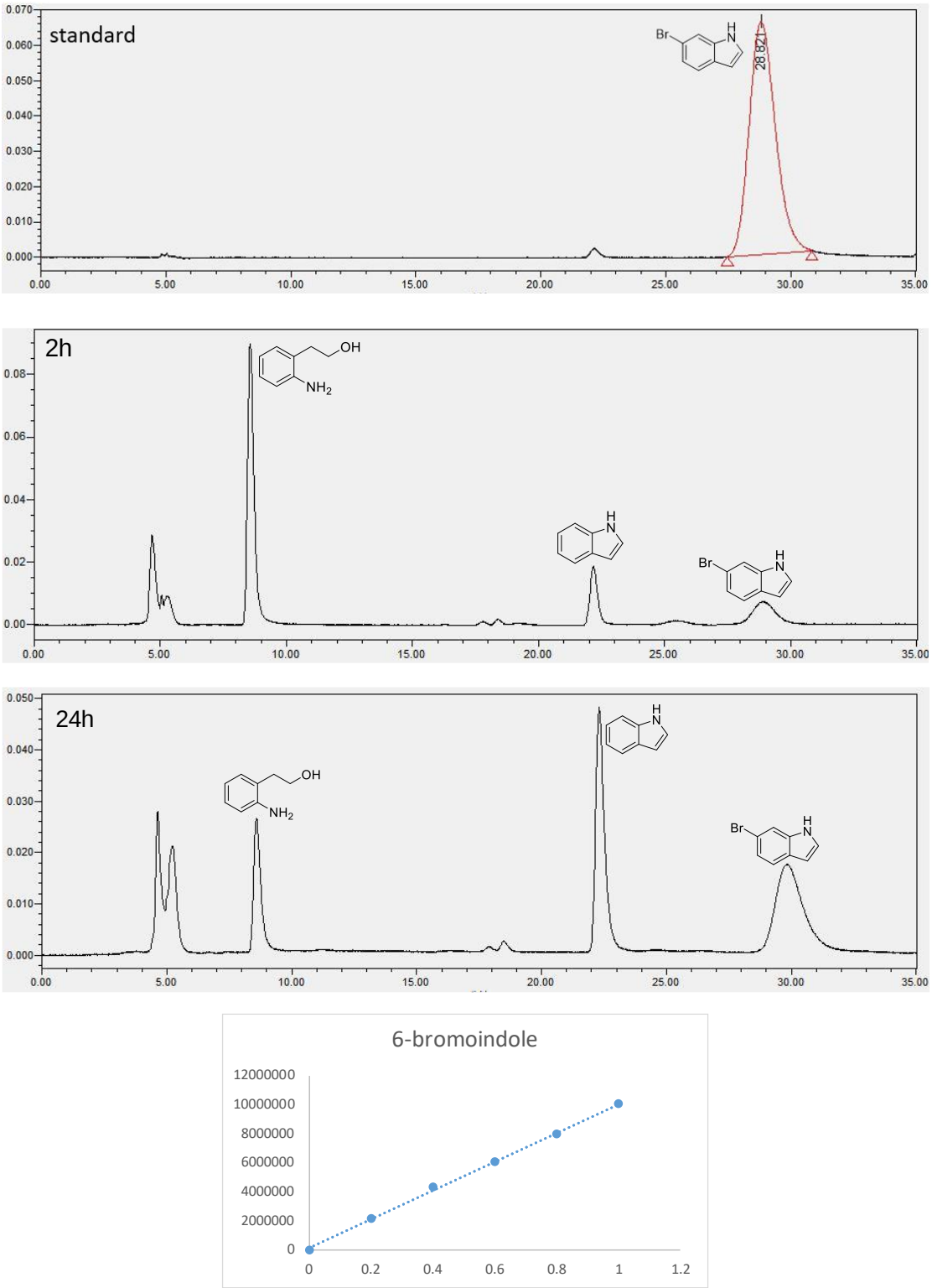


Fig. S13 Biosynthesis of 6-Br-indole via SttH/RebF coupled with HLADH. (HPLC method C). 1 mM 2-(2-aminophenyl)-ethanol, 0.1 mM NAD^+, 2.5 μM SttH, 10 μM FAD, 10 μM RebF, 2.5 μM HLADH, 650 U/mL Catalase, 40 mM NaBr, 50 mM KPi buffer (pH 7.5), 25 °C, 24 h.

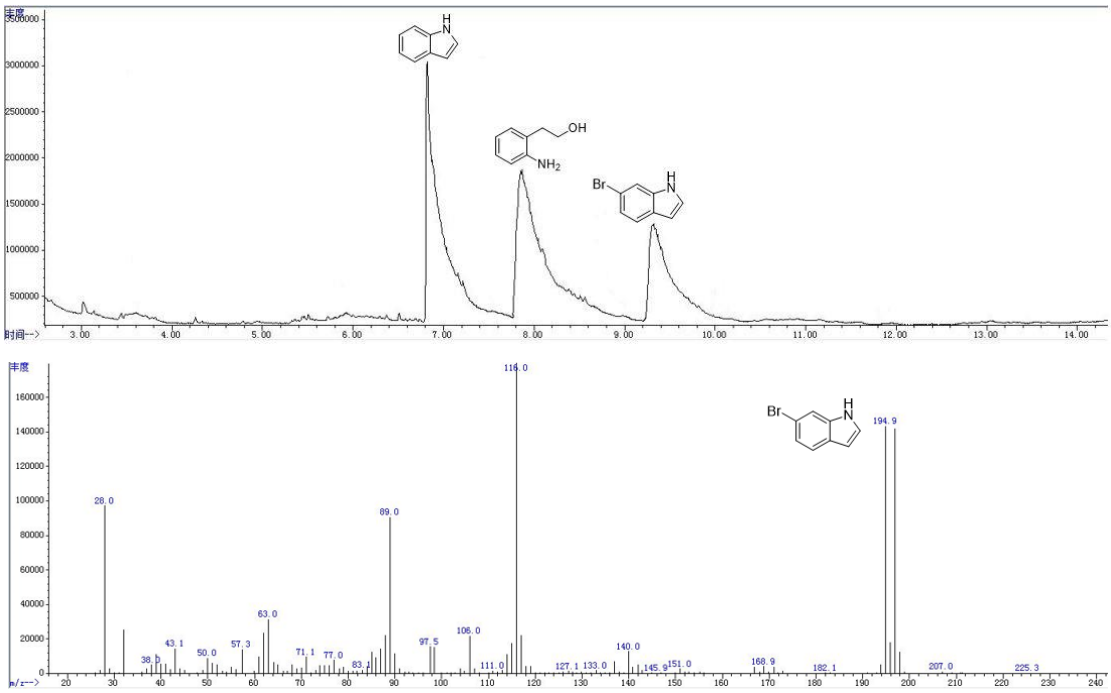
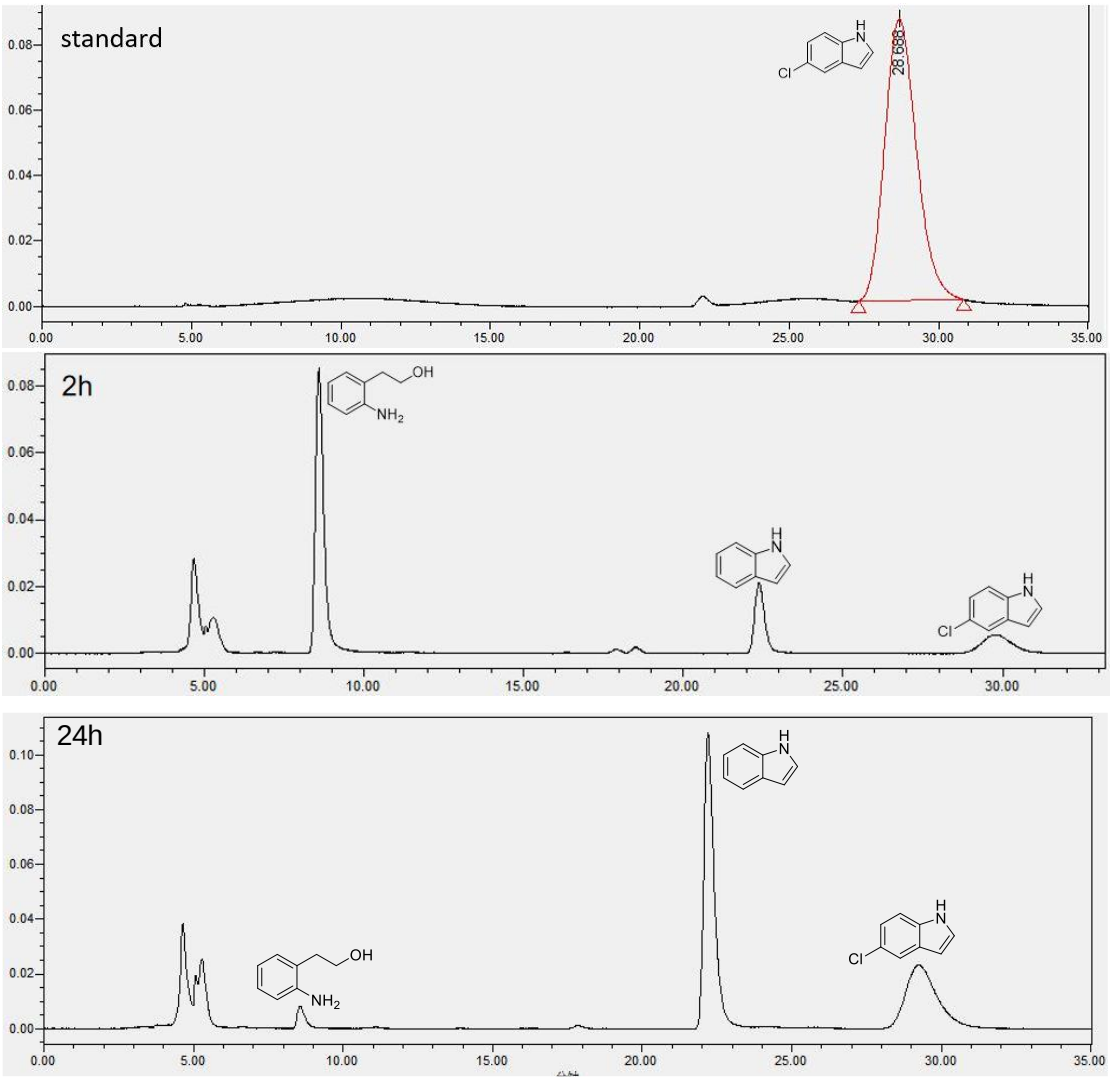


Fig. S14 Biosynthesis of 6-Br-indole via SttH/RebF coupled with HLADH. (GC-MS)



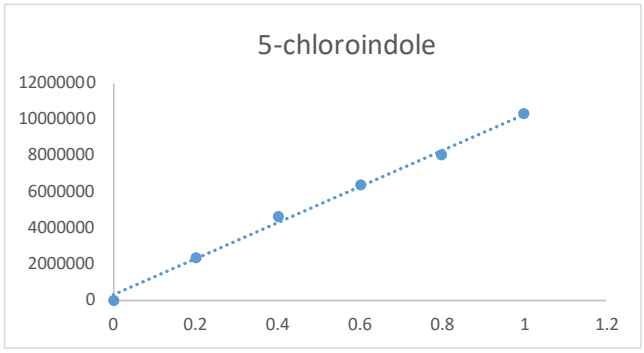


Fig. S15 Biosynthesis of 5-Cl-indole via *Xszen*FHal/RebF coupled with HLADH. (HPLC method C). 1 mM 2-(2-aminophenyl)-ethanol, 0.1 mM NAD^+, 2.5 µM *Xszen*FHal, 10 µM FAD, 10 µM RebF, 2.5 µM HLADH, 650 U/mL Catalase, 40 mM NaCl, 50 mM KPi buffer (pH 7.5), 25 °C, 24 h.

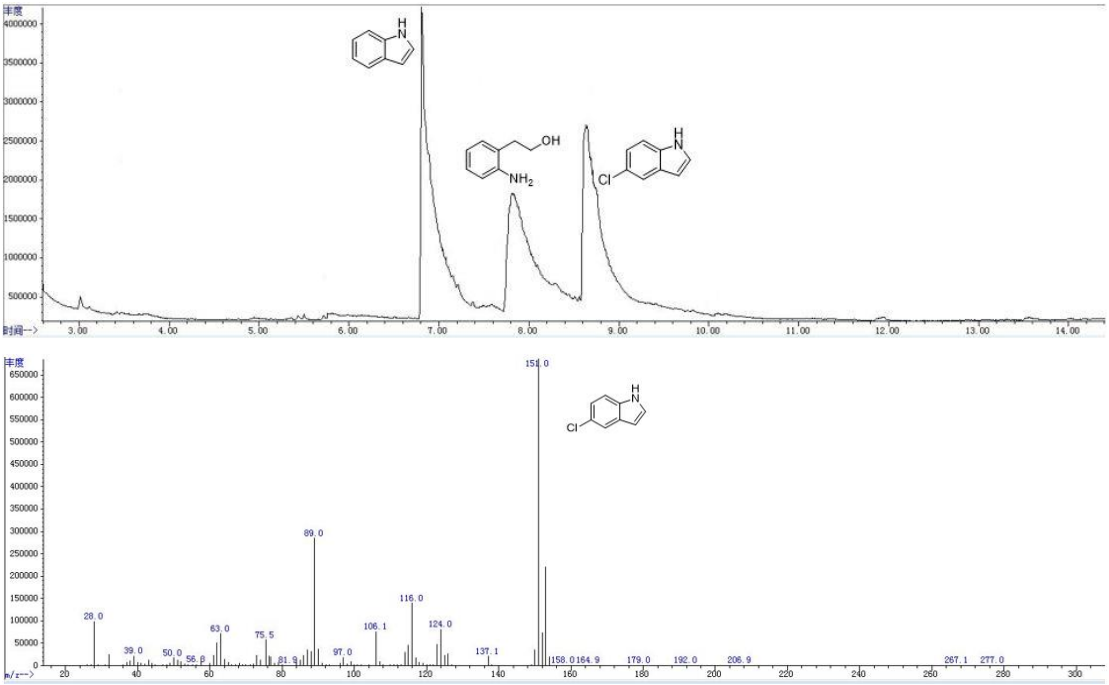


Fig. S16 Biosynthesis of 5-Cl-indole via *Xszen*FHal/RebF coupled with HLADH (GC-MS).

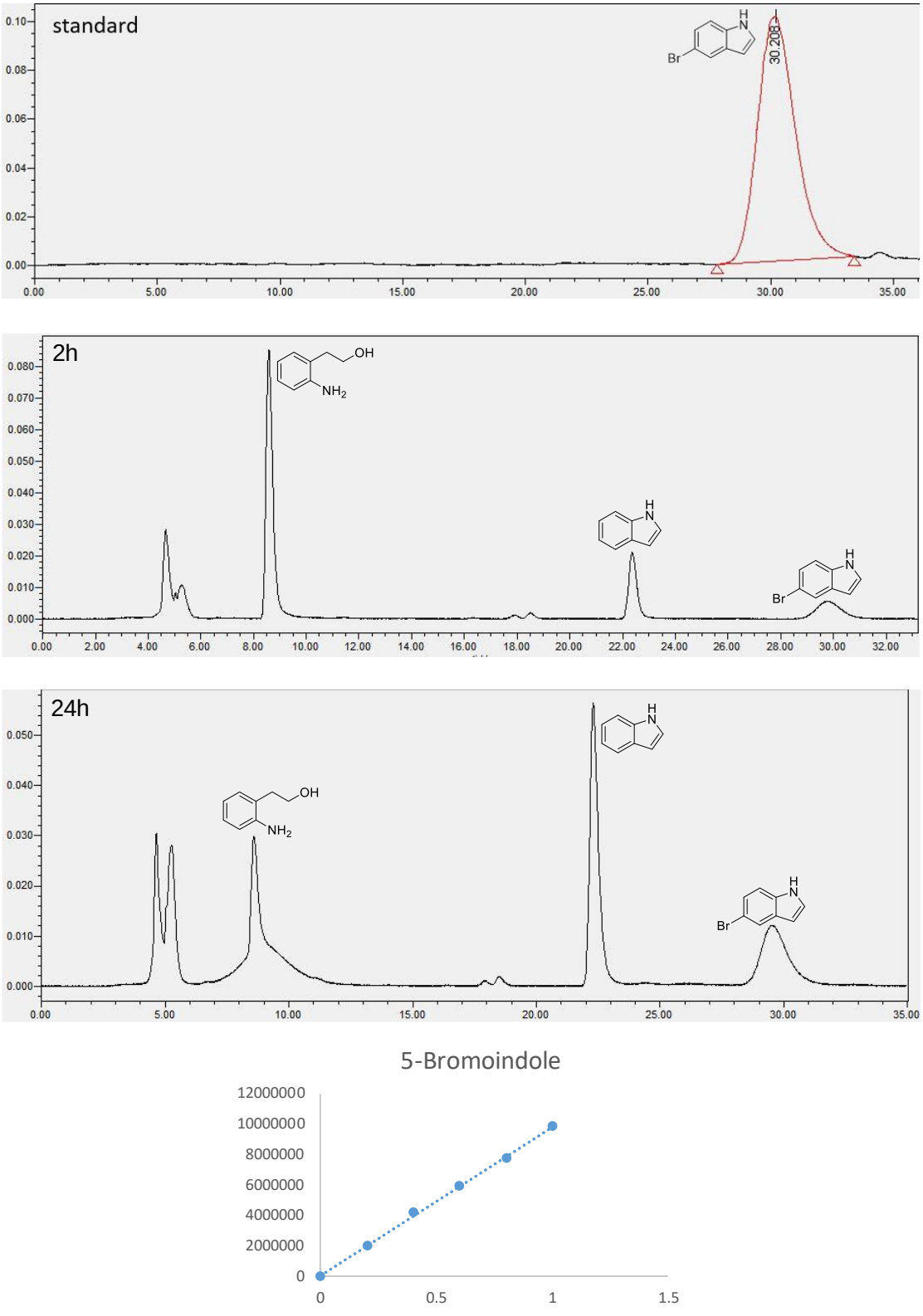


Fig. S17 Biosynthesis of 5-Br-indole via *Xszen*FHal/RebF coupled with HLADH (HPLC method C). 1 mM 2-(2-aminophenyl)-ethanol, 0.1 mM NAD^+, 2.5 µM *Xszen*FHal, 10 µM FAD, 10 µM RebF, 2.5 µM HLADH, 650 U/mL Catalase, 40 mM NaBr, 50 mM KPi buffer (pH 7.5), 25 °C, 24 h.

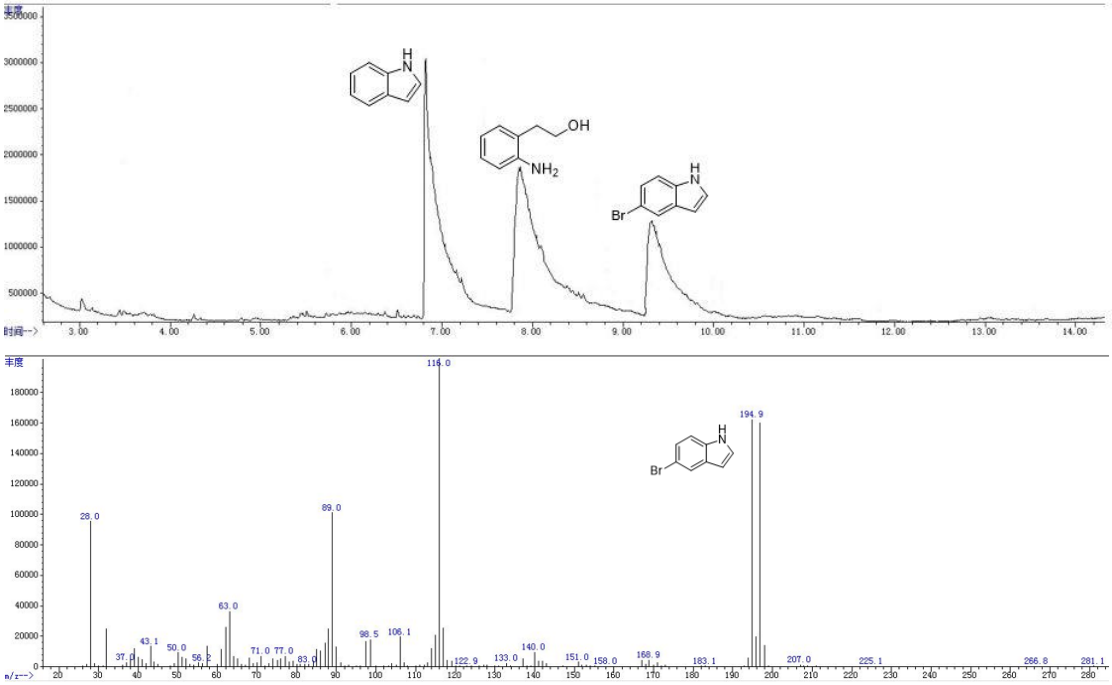


Fig. S18 Biosynthesis of 5-Br-indole via *Xszen*FHal/RebF coupled with HLADH (GC-MS).

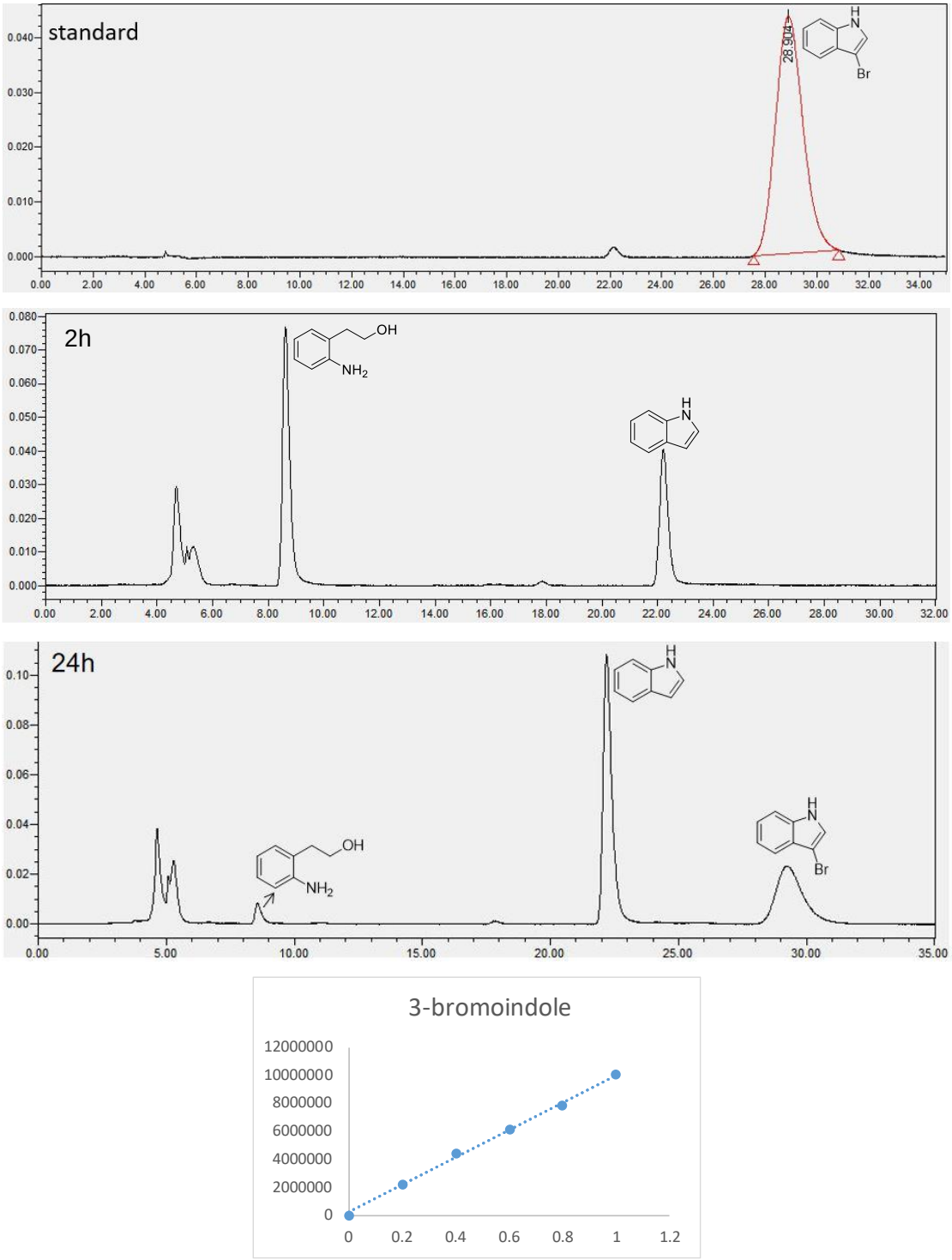


Fig. S19 Biosynthesis of 3-Br-indole via BrvH/RebF coupled with HLADH (HPLC method C). 1 mM 2-(2-aminophenyl)-ethanol, 0.1 mM NAD^+, 2.5 μM BrvH, 10 μM FAD, 10 μM RebF, 2.5 μM HLADH, 650 U/mL Catalase, 40 mM NaBr, 50 mM KPi buffer (pH 7.5), 25 °C, 24 h.

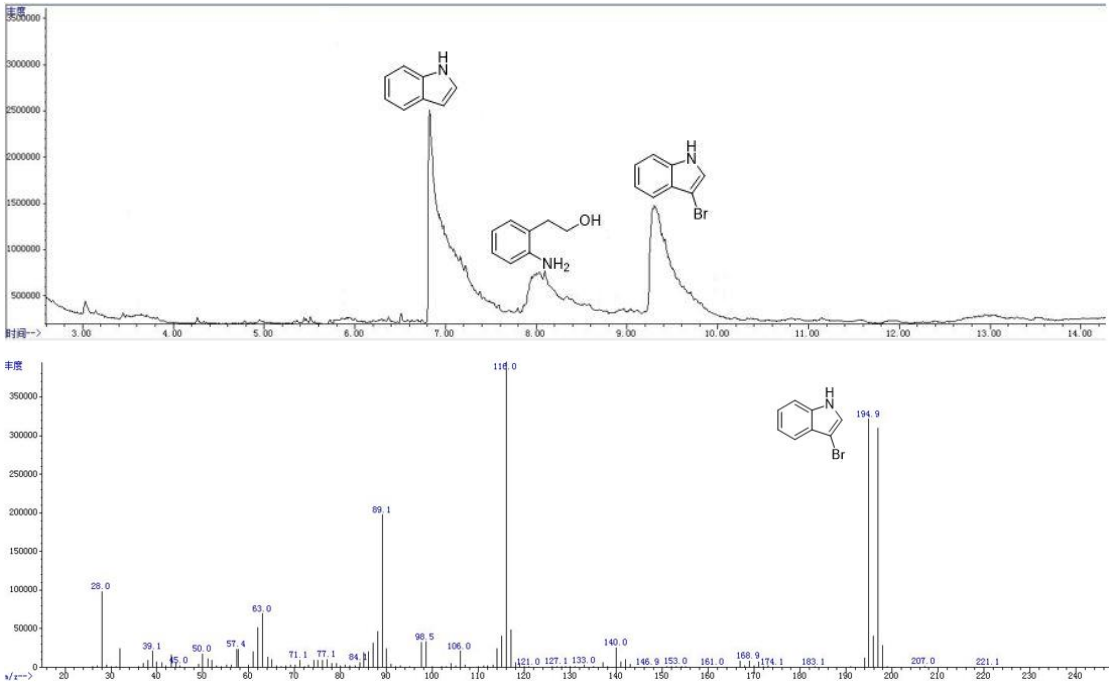


Fig. S20 Biosynthesis of 3-Br-indole via BrvH/RebF coupled with HLADH (GC-MS).

The Figures of NMR

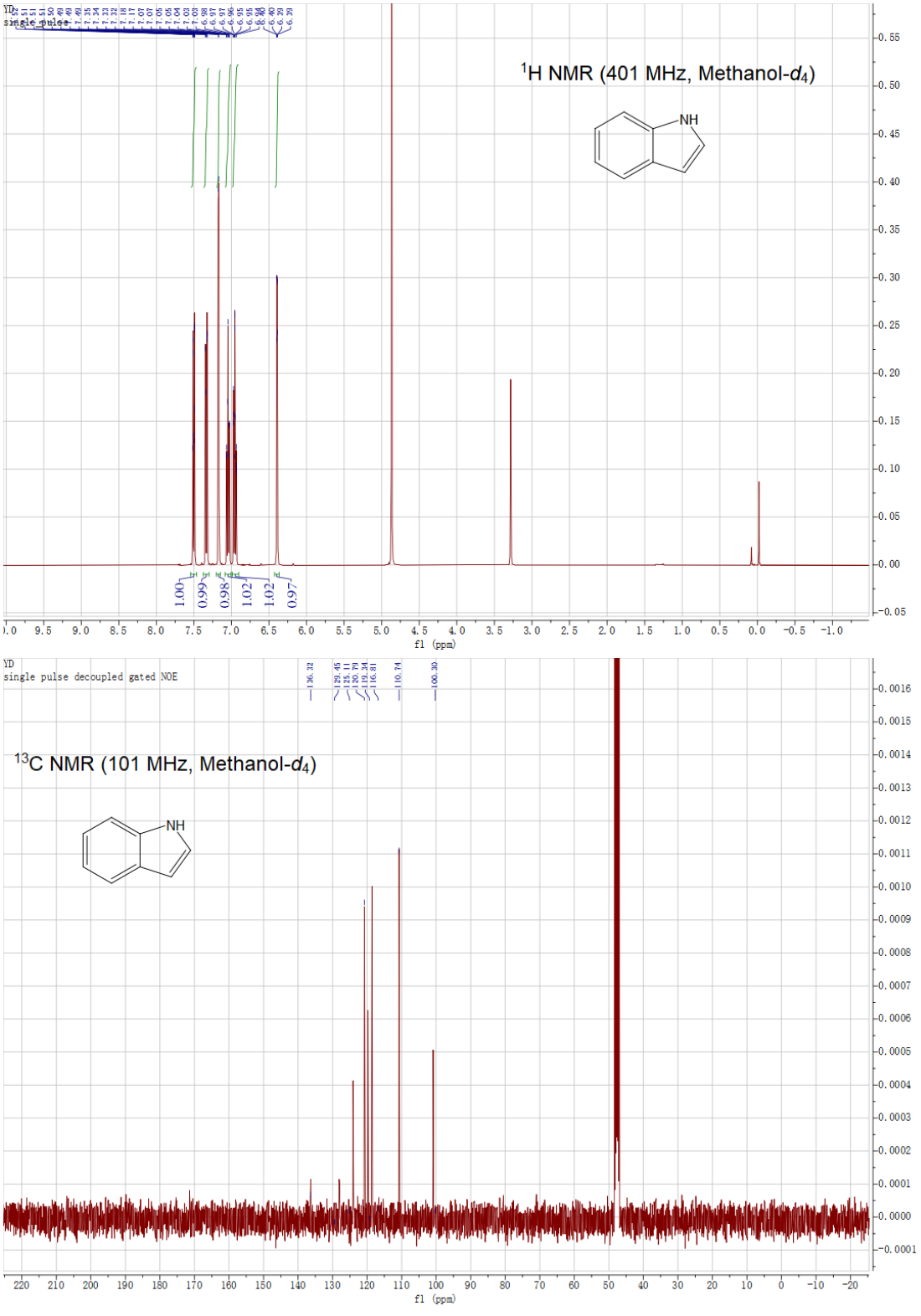


Fig. S21 The NMR spectrum of indole (**1b**).

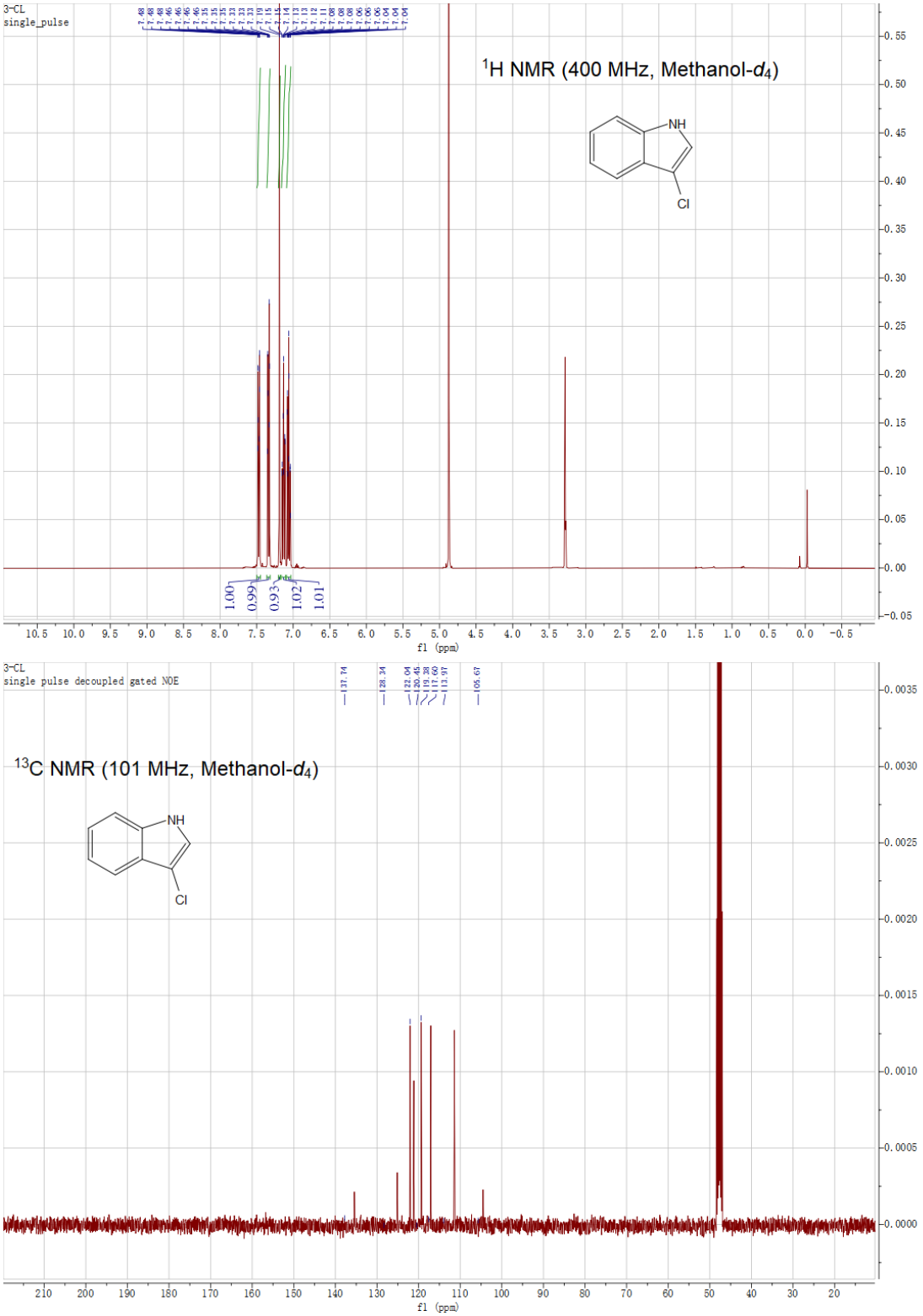


Fig. S22 The NMR spectrum of 3-Cl-indole (**1c**).

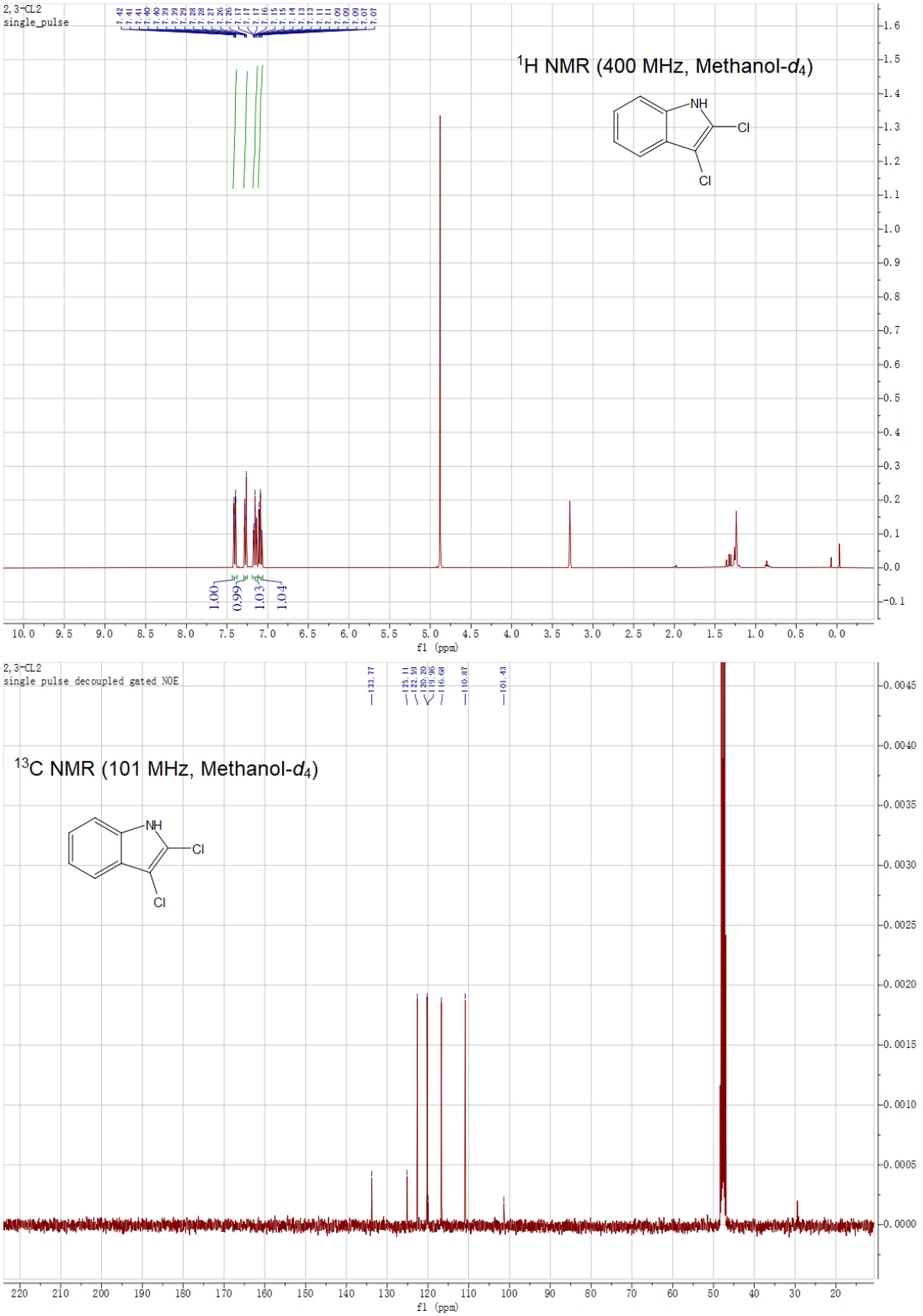


Fig. S23 The NMR spectrum of 2,3-dichloroindole (**1d**).

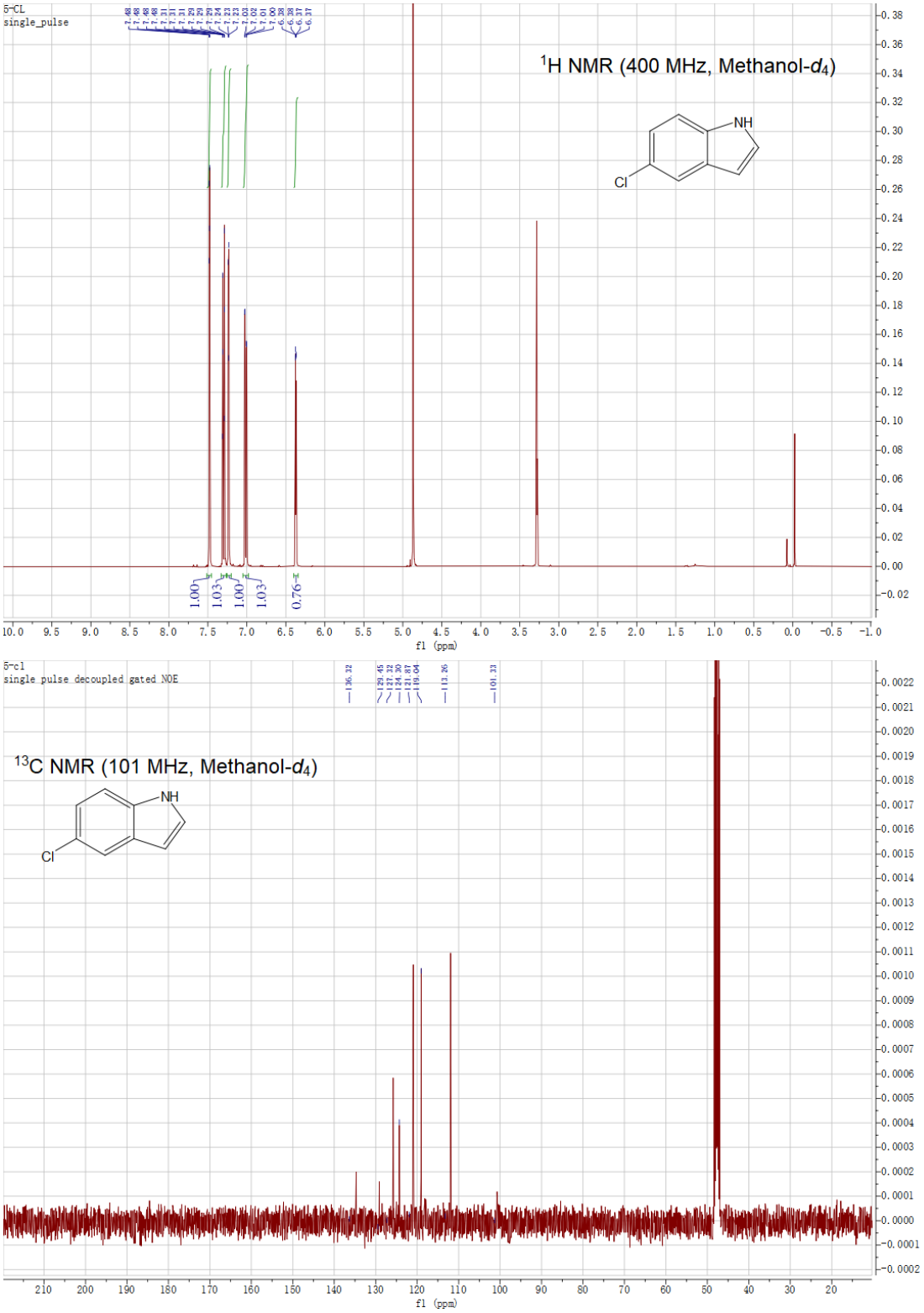


Fig. S24 the NMR spectrum of 5-Cl-indole (**1e**).

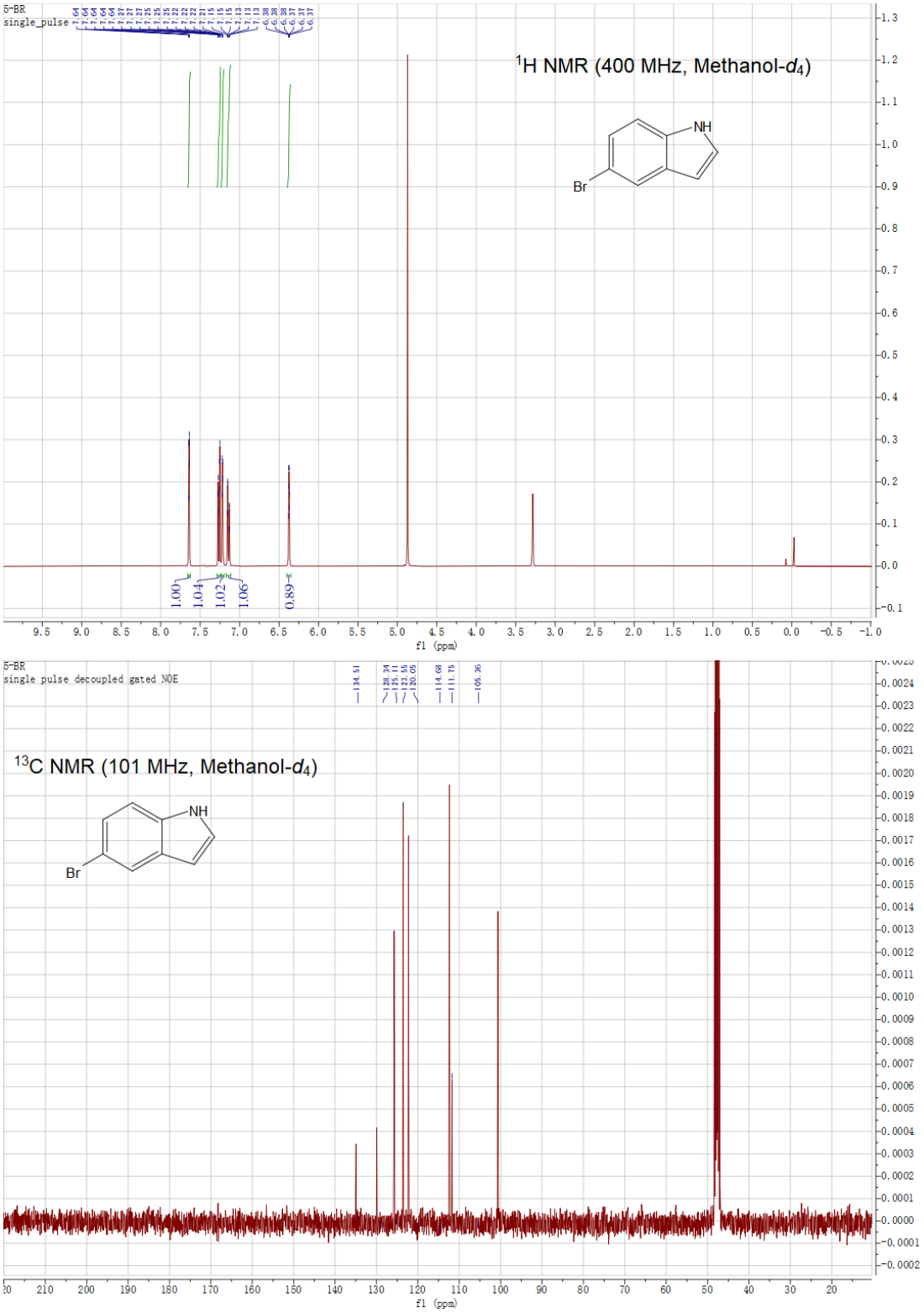


Fig. S25 the NMR spectrum of 5-Br-indole (**1f**).

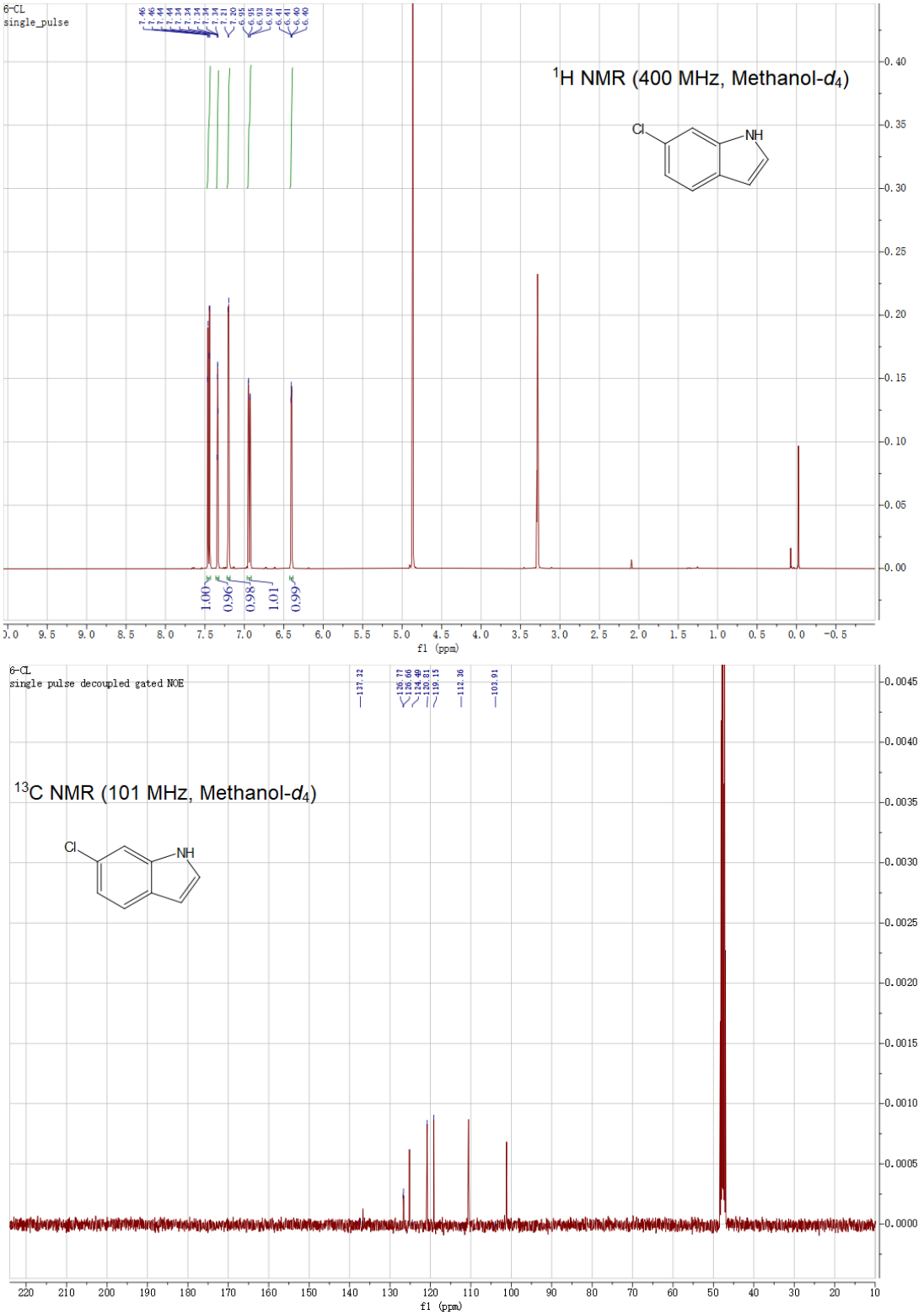


Fig. S26 the NMR spectrum of 6-Cl-indole (**1g**).

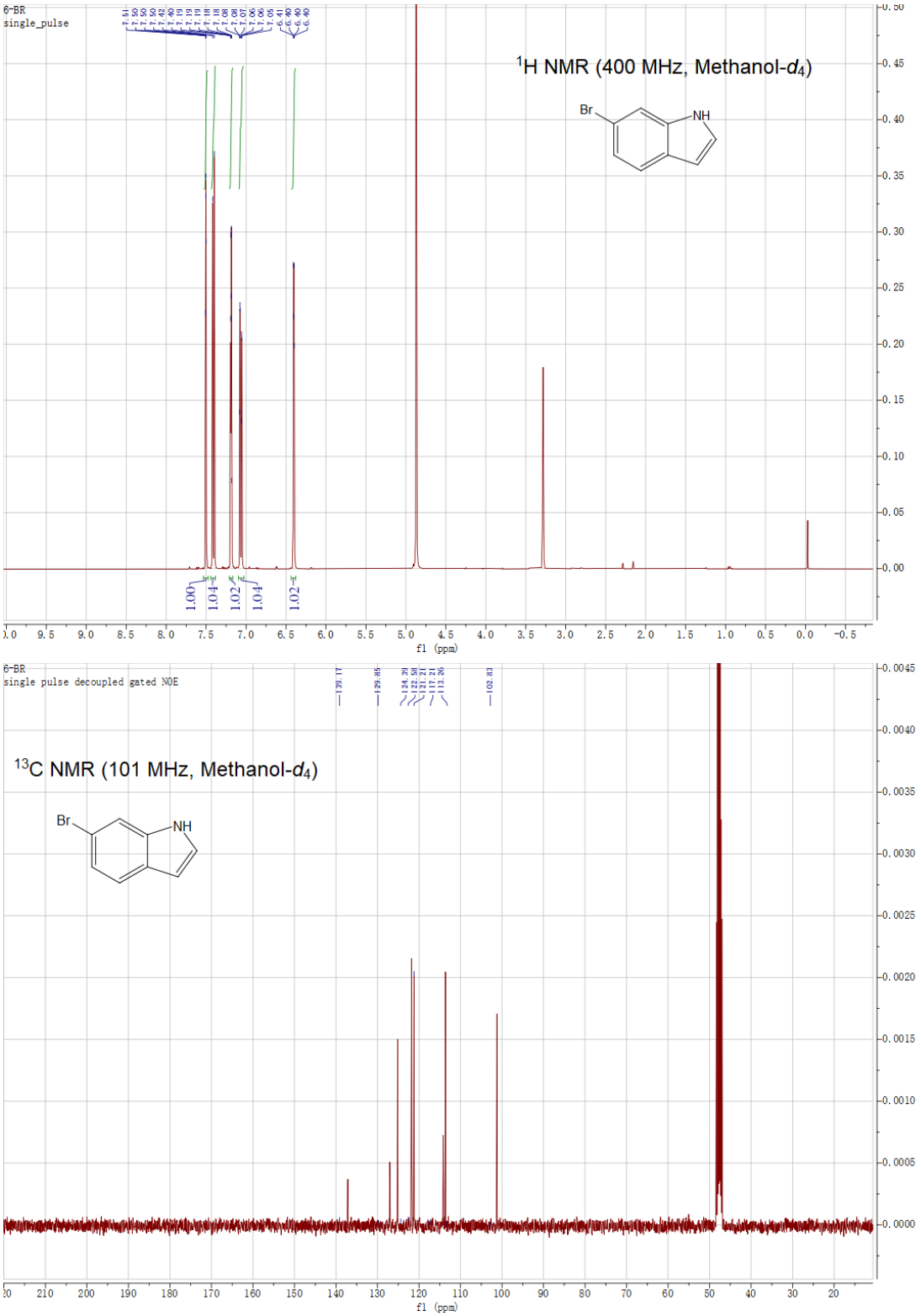


Fig. S27 the NMR spectrum of 6-Br-indole (**1h**).

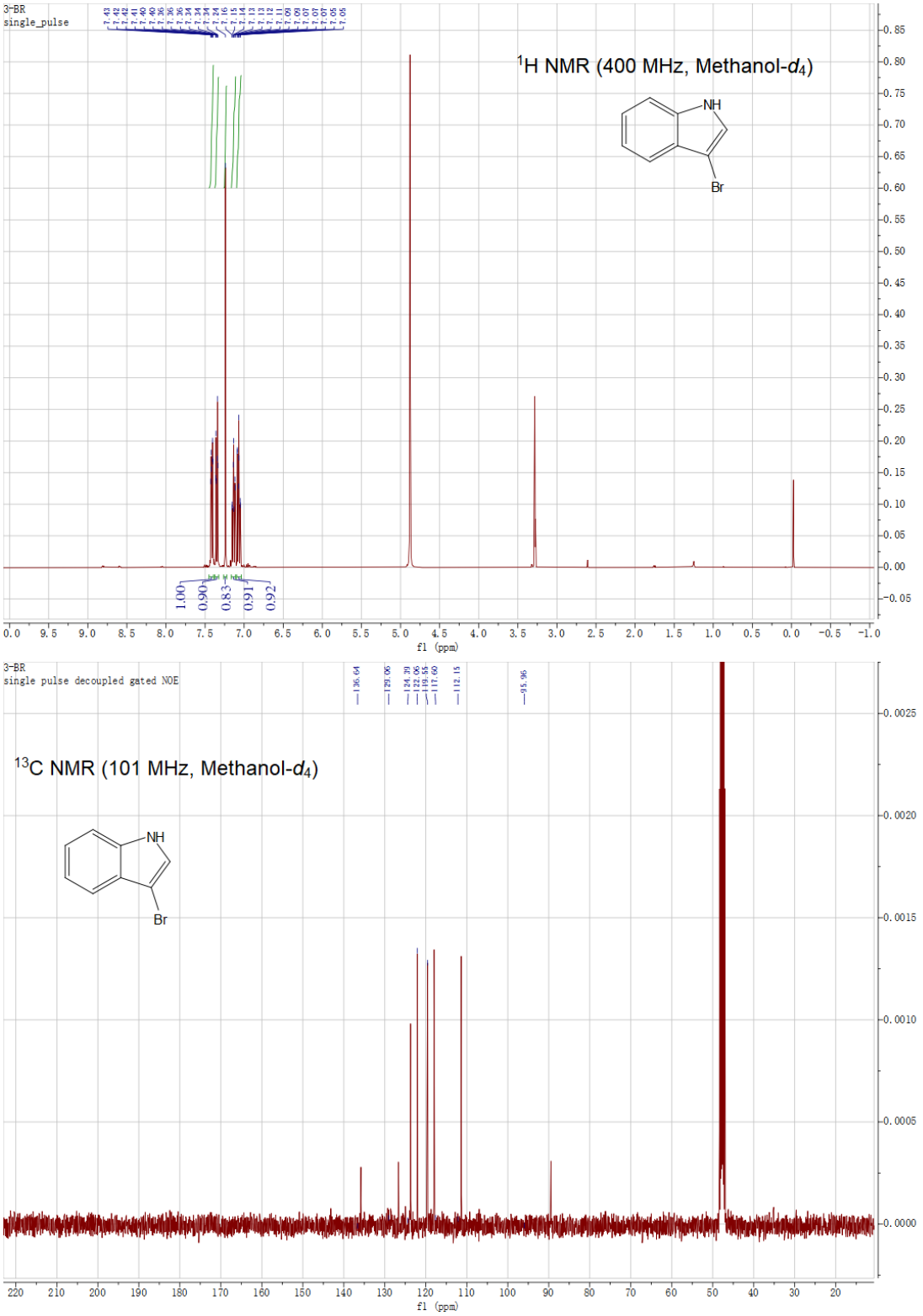


Fig. S28 the NMR spectrum of 3-Br-indole (**1i**).

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