Supporting Information

Complete stereoinversion of L-tryptophan by a fungal single-module nonribosomal peptide synthetase

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1. Materials and Methods

1.1. Chemicals and general methods

L-Tryptophan is purchased from Fisher Chemicals. D-Tryptophan is purchased from Acros Organics. *N*-acetyl-L-tryptophan and *N*-acetyl-D-tryptophan are purchased from TCI. N_α-Boc-L-tryptophan-*N*-hydroxy-succinimide ester, N_α-Boc-D-tryptophan-*N*-hydroxy-succinimide ester, and all other tryptophan amino acid derivatives are purchased from Chem-Impex Int'l. Inc. Isopropyl-β-D-1-thio-galactopyranoside (IPTG) was purchased from Carbosynth. Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP-HCl) was purchased from GoldBio Biotechnology. All other chemicals were purchased from Sigma-Aldrich. PCR reactions were performed using the Phusion® high-fidelity DNA polymerase (New England Biolabs) and used according to the manufacturer's instructions. Custom oligonucleotides were synthesized by Integrated DNA Technologies. *Escherichia coli* strain DH10B was used for cloning procedures.

1.2. Protein expression and purification

The *ivoA* gene (AN10576) exon fragments were cloned from the genomic DNA extract of *A. nidulans* ΔEM strain,[1] and assembled through yeast homologous recombination using a Frozen-EZ Yeast Transformation II Kit (Zymo research). Gene fragments were integrated into a 2μ-based yeast expression vector (pXW55) with uracil auxotrophic marker and ADH2 promoter and terminator. To facilitate purification, the target gene was fused with an octahistidine tag at its *N*-terminus. The full-length wild-type IvoA and mutants were expressed in *S. cerevisiae* JHY686 strain and expression was autoinduced in YPD medium. Briefly, single colonies of yeast cells harboring plasmids was inoculated into SDCt uracil drop-out culture and left grown at 28 °C for 2days. The seed culture was then inoculated into YPD culture (1 ml to 50 mL) and left grown at 28 °C for another 2 days. Cells were harvested by centrifugation and washed once with cell lysis buffer (50 mM K_2HPO_4 (pH 7.5), 10 mM imidazole, 300 mM NaCl, 5% glycerol). Cells were flash frozen in liquid nitrogen and lysed by using a stainless-steel Waring blender. The cell lysate was cleared by centrifugation at 26,000 g for 60 min at 4 °C and the supernatant was filtered through a 0.22 μm filter (Millipore). The filtrate was incubated with Ni^{2+}-NTA resin for 30 min at 4 °C and then the slurry was loaded onto a gravity column. The resin was washed and eluted with increasing concentrations of imidazole in cell lysis buffer. The fractions were examined by SDS-PAGE gels and targeted proteins were subject to size-exclusion chromatography by using a HiLoad Superdex 200 26/60 column (GE Healthcare) equilibrated in storage buffer [50 mM K_2HPO_4 (pH 7.5), 150 mM NaCl, 1 mM TCEP]. Pure fractions were concentrated to 20 mg/mL by Amicon concentrators (Millipore), supplemented with 10% glycerol and stored at -80 °C. Protein concentrations were determined by Bradford assay.

For individual domain expression, the expression plasmids were constructed by subcloning the corresponding domain region into a modified pET28a (+) vector (Addgene plasmid #29656). The resulting *N*-terminal TEV protease cleavable hexahistidine tagged individual domains were overexpressed in *E. coli* BL21(DE3) cells in LB medium in the

presence of 50 mg/L kanamycin. Expression was induced by 100 μM IPTG when OD_{600} reached 1.0 and the cell cultures were left grown at 16 °C overnight. Cells were harvested by centrifugation and lysed by sonication. Purification was performed similarly to the full-length protein.

1.3. Fermentation product isolation and purification

The fermentation product was analyzed with a Shimadzu 2020 LC-MS (Phenomenex Kinetex, 1.7 μm, 2.0 X 100 mm, C18 column) using positive and negative mode electrospray ionization with a linear gradient of 5-95% MeCN-H_2O supplemented with 0.1% (v/v) formic acid in 15 min followed by 95% MeCN for 3 min with a flow rate of 0.3 mL/min. For structural characterization, *N*-acetyl-D-tryptophan and D-tryptophan were isolated from a 2L yeast culture overexpressing IvoA protein. The cell pellets containing D-tryptophan were removed by centrifugation and the supernatant containing N-acetyl-D-tryptophan was collected separately.

To purify *N*-acetyl-D-tryptophan, the pH value of the supernatant was adjusted to 3 by using 1M HCl. The acidified supernatant was extracted with ethyl acetate and the organic layer was combined. The organic solvent was removed by rotavap and the crude extract was dried over Na_2SO_4. *N*-Acetyl-D-tryptophan was purified by silica-gel chromatography. Fractions containing the target compound were combined and further purified by semipreparative HPLC using a reverse-phase column (Phenomenex Kinetics, C18, 5 μm, 100 Å, 250 x 4.6 mm). The planar structure of *N*-acetyl-D-tryptophan was confirmed by comparing NMR spectrum with spectrum reported in the literature and database.[3] ^{1}H-NMR (500 MHz, CD_3OD): δ 1.89 (s, 3H), 3.15 (dd, *J* = 14.7, 7.5 Hz, 1H), 3.35 (dd, overlap with solvent, 1H), 4.69 (t, *J* = 14.7, Hz, 1H), 7.00 (ddd, *J* = 8.0, 7.0, 1.0 Hz, 1H), 7.07 (m, 2H), 7.31 (dt, *J* = 8.1, 0.9 Hz, 1H), 7.56 (dt, *J* = 7.9, 1.0 Hz, 1H). The stereochemistry of *N*-acetyl-D-tryptophan was determined by chiral analytical HPLC with a CHIRALPAK® IA-3 (150 x 4.6 mm, 3 μm) at room temperature. The mobile phase was 80/20/0.1/0.1 hexanes/ethanol/TFA/DEA and the flow-rate was 1.0 mL/min.

To purify D-tryptophan, the cell pellet was extracted by acetone and the solvent was removed by rotavap. The crude residue was dissolved in mobile phase A (water containing 0.1 (v/v) TFA) and applied to reverse-phase flash-chromatography. Basically, 20 mL of Cosmosil 140 C_{18}-OPN resin (Nacalai Tesque, Inc.) was packed in a Luer-Lock, non-jacketed glass column (Sigma) and equilibrated with mobile phase A. The resin was washed with 3 column volume (CV) of mobile phase and then eluted with increasing methanol content in a step-wise manner. Tryptophan was eluted at 15-25% (v/v) methanol fractions. The pooled fractions were further purified by semipreparative HPLC using a reverse-phase column (Phenomenex Kinetics, C18, 5 μm, 100 Å, 250 x 4.6 mm). The planar structure of D-tryptophan was confirmed by comparing NMR spectrum with spectrum reported in the literature and database. ^{1}H-NMR (500 MHz, D_2O): δ 3.37 (dd, *J* = 15.4, 7.8 Hz, 1H), 3.51 (dd, *J* = 15.4, 5.0 Hz, 1H), 4.19 (dd, *J* = 7.7, 5.0 Hz, 1H), 7.20 (ddd, *J* = 8.0, 7.0, 1.1 Hz, 1H), 7.28 (ddd, *J* = 8.2, 7.0, 1.2 Hz, 1H), 7.32 (s, 1H), 7.54 (dt, *J* = 8.2, 1.0 Hz, 1H), 7.72(dt, *J* = 8.0, 1.0 Hz, 1H). Similarly, L-

tryptophan was purified from yeast cells without overexpressing ivoA protein. ^{1}H-NMR (500 MHz, D_2O): δ 3.40 (dd, *J* = 15.4, 7.6 Hz, 1H), 3.52 (dd, *J* = 15.4, 5.2 Hz, 1H), 4.26 (dd, *J* = 7.5, 5.0 Hz, 1H), 7.19 (t, *J* = 7.5 Hz, 1H), 7.28 (t, *J* = 7.6 Hz, 1H), 7.33 (s, 1H), 7.53 (d, *J* = 8.1 Hz, 1H), 7.71(d, *J* = 8.1 Hz, 1H). The stereochemistry was determined by chiral analytical HPLC with a Crownpak® CR(+) column (150 mm x 4 mm x 3.5 μm, Daicel) at room temperature. The mobile phase was aq. $HClO_4$ 1% (w/v) supplemented with 15% (v/v) MeOH and the flow rate was 1.0 ml/min.

1.4. Enzymatic assay.

The hydroxylamine-based colorimetric assay for adenylation activity was performed according to the literature.[2] Acetyltryptophan acetyltransferase activity was performed by incubating 1-100 μM IvoA with 1 mM D-tryptophan or other substrates with 1 mM acetyl-CoA or 1 mM acetyl-phosphate in 100 mM phosphate buffer (pH 7.5). The reaction mixture was incubated at room temperature and the reaction was quenched at different time interval by mixing with 5-fold volume of methanol. The mixture was clarified by centrifugation to remove protein and salts, and the supernatant was dried *in vaccuo* by using speedvac. The residue was dissolved in methanol and subjected to LC-MS analysis. For ATP-dependent acetyltransferase activity, 1 mM L/D-tryptophan, 5 mM ATP, 1 mM CoA and 5 mM $MgCl_2$ were used.

The ATP-dependent stereoinversion activity was typically performed with 2-5 μM IvoA, 1 mM L/D-tryptophan, 3 mM ATP and 10 mM $MgCl_2$ in 100 mM phosphate buffer (pH 7.5), and the reaction was quenched by mixing with 5-volume of methanol. The solvent was removed in vaccuo by speedvac and the residue was dissolved in ethanol and analyzed by chiral-HPLC by using a Crownpak® CR(+) column (150 mm x 4 mm x 3.5 μm, Daicel) at room temperature. The mobile phase was aq. $HClO_4$ 1% (w/v) supplemented with 15% (v/v) MeOH and the flow rate was 1.0 ml/min.

When assays were performed in D_2O, enzyme stock solution was buffer exchanged into K_2HPO_4 buffer in D_2O (pD 7.5) by using Zeba™ Spin Desalting Column (ThermoFisher Scientific). All substrates and cofactors were dissolved in the same buffer.

The L-/D-tryptophan loading reactions were performed by incubating 80 μM *holo*-IvoA-ΔC with 5 mM ATP, 10 mM $MgCl_2$ and 1 mM L-/D-tryptophan in a final volume of 50 μL. The reaction was allowed to proceed for 15 min before a two-fold dilution with mQH_2O and analysis by UHPLC-ESI-Q-TOF-MS.

The thioesterase activity assay of standalone IvoA-C was performed in ammonium acetate buffer (20 mM, pH = 6.9). Typically, 5 mM synthetic substrate (5% DMSO) was incubated with 50 μM enzyme. The reaction was analyzed by HPLC. Boiled enzyme was used as control to measure the background nonenzymatic hydrolysis.

The loaded IvoA-ΔC(E^0) was prepared enzymatically by incubating *holo*-enzyme with respective substrate (1 mM) in the presence of excess ATP (5 mM) and $MgCl_2$ (10 mM) in storage buffer for 2 min. The reaction was quenched by desalting the enzyme through Zeba™ Spin Desalting Column, which is equilibrated in the ammonium acetate buffer (20 mM, pH = 6.9). The desalted enzyme was immediately mixed with IvoA-C (50 μM), or boiled enzyme, or chemical

hydrolysis (1 M KOH). The hydrolysis reaction was quenched after 1 min by mixing with 2 volume of acetonitrile and subjected to LC-MS analysis.

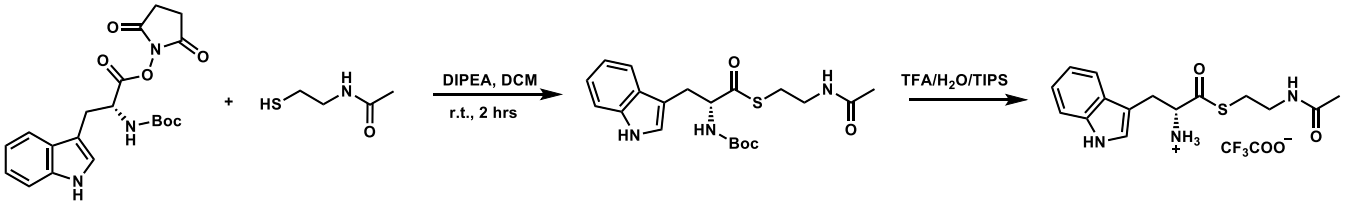
1.5. UHPLC-ESI-Q-TOF-MS Analysis of Intact Proteins

The L-/D-tryptophan loading reactions were analyzed on a Bruker MaXis II ESI-Q-TOF-MS connected to a Dionex 3000 RS UHPLC fitted with an ACE C4-300 RP column (100 x 2.1 mm, 5 μm, 30 °C). The column was eluted with a linear gradient of 5–100% MeCN containing 0.1% formic acid over 30 min. The mass spectrometer was operated in positive ion mode with a scan range of 200–3000 *m/z*. Source conditions were: end plate offset at −500 V; capillary at −4500 V; nebulizer gas (N_2) at 1.8 bar; dry gas (N_2) at 9.0 L min^{-1} ; dry temperature at 200 °C. Ion transfer conditions were: ion funnel RF at 400 Vpp; multiple RF at 200 Vpp; quadrupole low mass at 200 *m/z*; collision energy at 8.0 eV; collision RF at 2000 Vpp; transfer time at 110.0 μs; pre-pulse storage time at 10.0 μs.

1.6. Genetic manipulation

The *S. cerevisiae hpa3*Δ mutant strain derived from parent JHY686 strain was constructed by integration of a LEU2 marker to the *hpa3* loci through homologous recombination. The correct integration was selected by colony-PCR. The resulting strain JHY686-YH (*MATα lys2*Δ0 *his3*Δ1 *leu2*Δ0 *ura3*Δ0 *pep4*Δ *SAL1*$^+$ *HAP1*$^+$ *CAT5*(91M) *MIP1*(661T) *MKT1*(30G) *RME1* (INS-308A) *TAO3* (1493Q) *prb1*Δ::*ADH2p-npgA-ACS1t hpa3*Δ:: LEU2) was used to transform plasmid overexpressing IvoA protein.

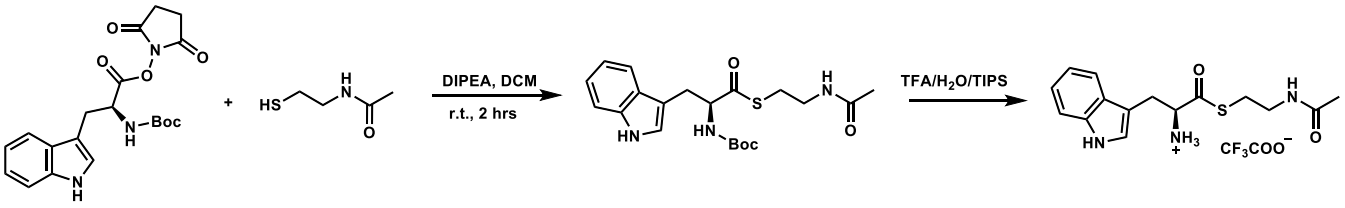
1.6 Synthesis of D-Trp-SNAC



N_α-Boc-D-tryptophan-*N*-hydroxy-succinimide ester (0.2 g, 0.5 mmol) was dissolved in anhydrous dichloromethane (10 mL) at room temperature, and to this solution was added *N*-acetylcysteamine (0.07 g, 0.6 mmol) and diisopropylethylamine (DIPEA, 0.12 g, 1 mmol). This was stirred at room temperature for 2 hrs and washed with saturated ammonium chloride. The organic layer was dried over sodium sulfate and removed by rotavap. The residue was subjected to silica flash chromatography. The resulting white solid product was dissolved in 2 mL of cocktail of 90% trifluoroacetic acid (TFA)/5% water/5% triisopropylsilane (TIPS) and stirred for 8 hrs. The solvents were evaporated to give a crude oil, which was taken up in minimal volume of dichloromethane and precipitated with diethyl ether. The resulting solid was further washed with diethyl ether to afford the final product in 80% yield. ^{1}H-NMR (d_6-DMSO, 500 MHz): δ 11.12 (s, 1H), 8.56 (s, 3H), 8.06

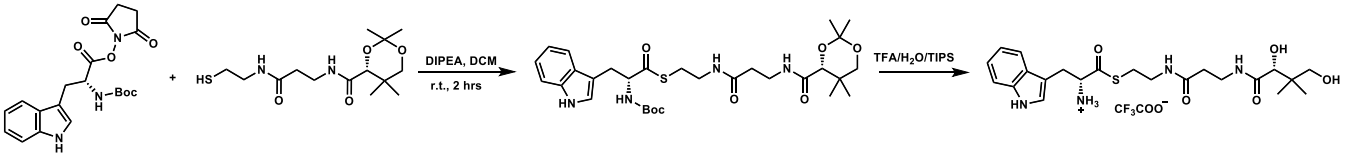
(t, 1H, J= 5.3 Hz), 7.55 (d, 1H, J= 7.7 Hz), 7.38 (d, 1H, J= 8.1 Hz), 7.25 (d, 1H, J= 2.5 Hz), 7.10 (ddd, 1H, J= 8.2, 7.0, 1.2 Hz), 7.02 (ddd, 1H, J= 8.0, 7.0, 1.1 Hz), 4.45 (t, 1H, J= 6.6 Hz), 3.27 (m, 2H), 3.15 (q, 2H, J= 6.6 Hz), 2.96 (td, 2H, J= 6.8, 3.0 Hz), 1.79 (s, 3H). ^{13}C-NMR (d_6-DMSO, 125 MHz): δ 196.5, 169.4, 136.3, 127.0, 125.2, 121.3, 118.7, 118.1, 111.7, 106.2, 59.0, 37.8, 28.4, 27.6, 22.6. HRMS ESI m/z calculated for $C_{15}H_{20}N_3O_2S^+$ $[M+H]^+$ 306.1271, found 306.1258.

1.7 Synthesis of L-Trp-SNAC



The synthesis of L-Trp-SNAC is essentially the same as D-Trp-SNAC, except N_α-Boc-L-tyrptophan-*N*-hydroxy-succinimide ester was used. ^{1}H-NMR (d_6-DMSO, 500 MHz): δ 11.14 (s, 1H), 8.61 (s, 3H), 8.06 (t, 1H, J= 6.2 Hz), 7.55 (d, 1H, J= 8.0 Hz), 7.38 (d, 1H, J= 8.1 Hz), 7.25 (s, 1H), 7.10 (t, 1H, J= 7.5 Hz), 7.02 (t, 1H, J= 7.5, 7.0, 1.1 Hz), 4.44 (t, 1H, J= 4.7 Hz), 3.28 (m, 2H), 3.14 (m, 2H), 2.96 (td, 6.7, 2.7, 2H), 1.80 (s, 3H). ^{13}C-NMR (d_6-DMSO, 125 MHz): δ 196.5, 169.5, 136.3, 127.0, 125.2, 121.3, 118.7, 118.1, 111.7, 106.2, 59.0, 37.8, 28.4, 27.6, 22.6. HRMS ESI m/z calculated for $C_{15}H_{20}N_3O_2S^+$ $[M+H]^+$ 306.1271, found 306.1264.

1.8 Synthesis of D-Trp-pant



N_α-Boc-D-tryptophan-*N*-hydroxy-succinimide ester (0.1 g, 0.25 mmol) was dissolved in anhydrous dichloromethane (5 mL) at room temperature, and to this solution was added dimethyl ketal protected pantetheine prepared (80 mg, 0.25 mmol)[3] and DIPEA, 0.06 g, 0.5 mmol). This was stirred at room temperature for 2 hrs and washed with saturated ammonium chloride. The organic layer was dried over sodium sulfate and removed by rotavap. The residue was subjected to silica flash chromatography. The resulting white-yellow solid was dissolved in 5 mL of cocktail of 75% trifluoroacetic acid (TFA)/20% water/5% triisopropylsilane (TIPS) and stirred for 24 hrs. The solvents were evaporated to give a crude oil, which was taken up in minimal volume of dichloromethane and precipitated with diethyl ether. The resulting solid was further washed with diethyl ether to afford the final product in total 60% yield. ^{1}H-NMR (d_6-DMSO, 500 MHz): δ 11.11 (s, 1H), 8.53 (s, 3H), 8.10 (t, 1H, J= 5.7 Hz), 7.72 (t, 1H, J= 6.1 Hz), 7.55 (d, 1H, J= 7.9 Hz), 7.38 (d, 1H, J= 8.1 Hz), 7.25 (d, 1H, J= 2.4 Hz), 7.10 (t, 1H, J= 7.5 Hz), 7.02 (t, 1H, J= 7.4 Hz), 4.45 (t, 1H, J= 6.7 Hz), 3.70 (s, 1H), 3.31 (m, overlap,

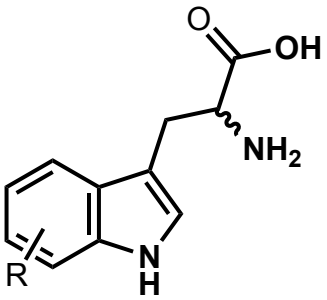
1H), 3.30 (m, overlap, 1H), 3.29 (m, 2H), 3.26 (m, 2H), 3.22 (m, overlap, 1H), 3.18 (m, overlap, 1H), 3.16 (m, 2H), 2.96 (m, 2H), 2.26 (t, 1H, J= 8.6 Hz), 0.80 (s, 3H), 0.78 (s, 3H). ^{13}C-NMR (d_6-DMSO, 125 MHz): δ 196.5, 172.9, 170.7, 136.3, 126.9, 125.2, 121.3, 118.7, 118.1, 111.6, 106.1, 75.0, 68.0, 59.0, 39.1, 37.7, 35.2, f35.1, 34.8, 28.3, 21.0, 20.3 HRMS ESI m/z calculated for $C_{22}H_{33}N_4O_5S^+$ $[M+H]^+$ 465.2166, found 465.2193.

2. Tables

Table S1. Oligomers used in this study

Name	Sequence (5'-3')
ivoA-FL-F1	ATATGGCTAGCCATCACCATCACCATCACCATCACACTGCCTCACCCAT CATCCAGCCAG
ivoA-FL-R1	CTTTGAGACAAATGGCATTCTGGTTGACCAGAGATCGGACTTC
ivoA-FL-F2	CGATCTCTGGTCAACCAGAATGCCATTTGTCTCAAAGTACTCTACAATG CC
ivoA-FL-R2	GATAATGAAAACTATAAATCGTGAAGGCATTCAAATGATTTCACACACCA TGCGCTCAAC
ivoA-ΔC-R	CTTGATAATGAAAACTATAAATCGTGAAGGCATTTACGACGATAACTTCG CGCTGAGATC
ivoA-S785A-F	GTAACGGCGGTGACGCAATAATGGCCATGGAGGC
ivoA-S785A-R	GCCTCCATGGCCATTATTGCGTCACCGCCGTTAC
ivoA-H963A-F	CTCTGTGTTCACGCCTTGGTCGTCGACTTC
ivoA-H963A-R	GAAGTCGACGACCAAGGCGTGAACACAGAG
ivoA-H1428A-F	GCGAATTGGAATTTAGTGCTACCATTATAGACGCTG
ivoA-H1428A-R	CAGCGTCTATAATGGTAGCACTAAATTCCAATTCGC
ivoA-T-F	GAAAACCTGTACTTCCAATCCAATTCAGATCCATCAGACAGCATGGTAG CG
ivoA-T-R	TTCGGATCCGTTATCCACTTCCAATTTAGGCTGAAGTTTTGGTTGCCAT TTCGCCAATC
ivoA-C-F	ACCTGTACTTCCAATCCAATGGTGTTTGCATTGAGCGCGATGTC
ivoA-C-R	ATCCGTTATCCACTTCCAATTCAAATGATTTCACACACCATGCGCTCAAC
Hpa3KO-F1	CACCTTTTGCAAGCACGAAGAACGTAGGCTCGAGGAGAACTTCTAGTAT ATCTACATACC
Hpa3KO-R1	AGTTATCTCTATACACAGTAGTCTACATTACACAGCGGTTTCGACTACG TCGTTAAGGCC
Hpa3KO-F2	GATGAGTGCAGACTAATGAAAAAGACCCCAGACCCATCTCCACCTTTTG CAAGCACGAAG
Hpa3KO-R2	CTTCGCAAGTGAGCCTATAGAAGCTAGAGTTATCTCTATACACAGTAGT CTACATTACAC
Hpa3KO-checkF	CCAATCCGGTTATTAAATCGTTATCAGGCGGAAACCTTC
Hpa3KO-checkR	CTTGCGATTAAATCGGAGAACAAACTATGAAAAGCCAATAAGAAAC

Table S2. Chiral HPLC retention time for substituted tryptophan analogues.



R	D-isomer t_R (min)	L-isomer t_R (min)
H	14.9	24.9
5-OMe	16.9	25.1
5-CN	19.5	33.2
5-NO_2	46.2	108.8
4-F	31.2	47.8
5-F	22.9	38.7
6-F	22.1	32.2
5-Cl	76.0	169.3
6-Cl	72.5	105.6
5-Br	140.2	345.4
6-Br	110.1	168.2
7-Br	74.5	102.4
2-Me	20.8	26.2
4-Me	38.2	57.9
5-Me	39.2	58.3
6-Me	37.8	54.5
7-Me	36.0	53.1

Table S3. Detailed figure captions for all figures in the main text.

Figure	Figure Caption
Figure 1	Diverse functions of single-module NRPS and NRPS-like enzymes. (A) Characterized examples. TqaB activates 2-aminoisobutyrate (AIB) and its C domain catalyzes enantioselective annulation of AIB to the indole ring of oxyfumiquinazoline F. TdiA activates indoel pyruvic acid (IPA) and its TE domain catalyzes head-to-tail dual Claisen condensation reactions. HqlA activates L-tyrosine and its R domain catalyzes reduction to yield tyrosyl aldehydes which further dimerize into 2,5-dihydropyrazine (B) IvoA studied in this work. IvoA was proposed to acetylate L-tryptophan. The P450 IvoC was proposed to install the hydroxy group at 6-postion of *N*-acetyl-L-tryptophan. The laccase IvoB was proposed to further oxidize *N*-acetyl-6-hydroxy-L-tryptophan for pigment formation. In this study, we demonstrate that IvoA does not catalyze acetylation. Instead, IvoA catalyzes ATP-dependent stereoinversion of L-tryptophan.
Figure 2	Characterization of IvoA activity. (A) Stereochemistry determination for isolated *N*-acetyl-D-tryptophan. The enantiomers were separated by using a CHIRALPAK® IA-3 (150 x 4.6 mm, 3 μm) at room temperature. The mobile phase was 80/20/0.1/0.1 hexanes/ethanol/TFA/DEA and the flow-rate was 1.0 mL/min. (B) Mass spectrometry shows the mass shift of tryptophan when the assay was performed in D2O. C) ^{1}H-NMR spectra indicate incorporation of deuterium at the α position: 1) the change of splitting pattern of the diastereotopic β proton signal due to smaller coupling constant ($^{3}J_{H\text{-}D}$)); 2) the disappearance of α proton signal, which is complicated by the overlapping methylene proton from emerging AMP product . D) Chiral HPLC resolution of tryptophan enantiomers from IvoA reaction demonstrated complete stereoinversion of L-tryptophan to D-tryptophan.
Figure 3	Working model of IvoA. IvoA can adenylate both L-tryptophan and D-tryptophan albeit the former is the preferred substrate. The activated tryptophan is tethered to the phosphopantetheine (Ppant) arm from T domain, which then delivers the corresponding tryptophanyl-S-Ppant thioester to E domain for epimerization to establish a mixture of DL-tryptophanyl-S-Ppant thioesters. C domain stereoselectively hydrolyzes D-tryptophanyl-S-Ppant thioester to release D-tryptophan.
Figure 4	Characterization of IvoA-C activity in vitro by LC-MS. Enzymatically loaded D/L-tryptophanyl-S-IvoA-ΔC(E^0) was mixed with standalone C domain at pH 6.9. The reaction was quenched after 1 min and the release free tryptophan amino acids were quantified by LC-MS. Boiled and mutant enzymes are used as negative control. Chemical hydrolysis by KOH is used as positive control.

3. Figures.

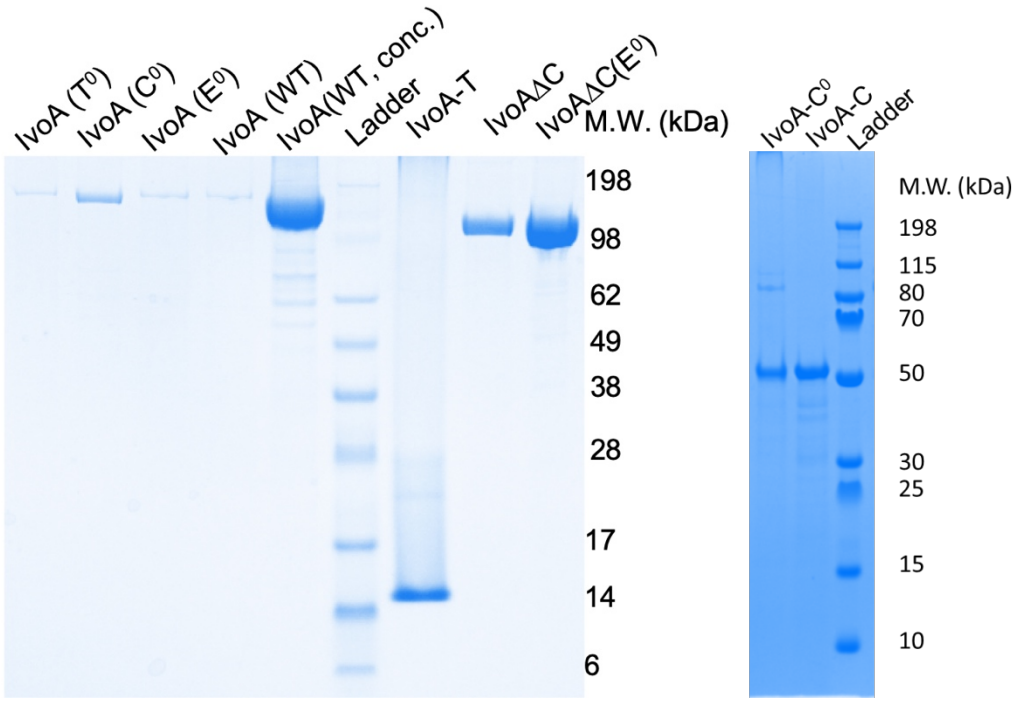


Fig. S1. SDS-PAGE analysis of purified IvoA proteins.
NuPAGE™ 4-12% Bis-Tris protein gels were used for analysis and gels were running with NuPAGE™ MES SDS running buffer.

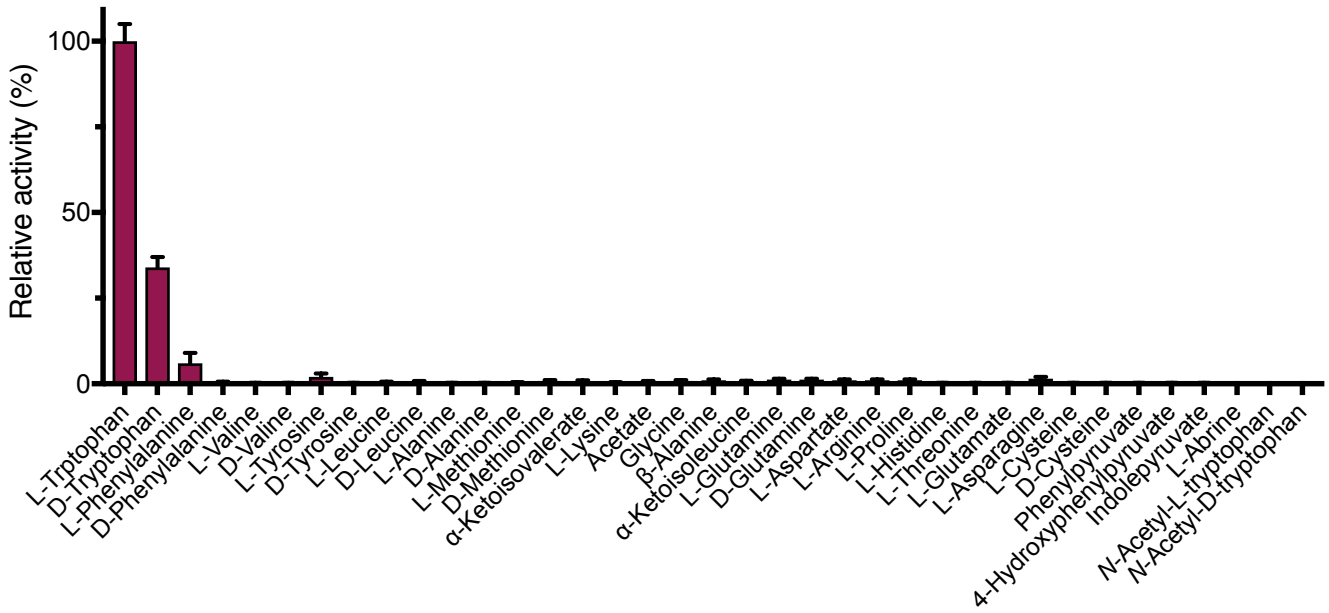


Fig. S2. Hydroxylamine-based colorimetric assay for studying substrate specificity of IvoA A domain.
The reaction condition is 2 μM of IvoA-T^0 + 3 mM of ATP + 0.1 mM of carboxylic acid substrate + 15 mM hydroxylamine + 15 mM $MgCl_2$ in Tris buffer (pH 8.0). The reaction is quenched after 3 hrs by addition of equivalent volume (150 μL) of stopping solution [10% (w/v) $FeCl_3 \cdot H_2O$ and 3.3% (w/v) trichloroacetic acid dissolved in 0.7 M HCl]. The precipitated enzyme was removed by centrifugation and the supernatant was measured for its absorbance at 540 nm.

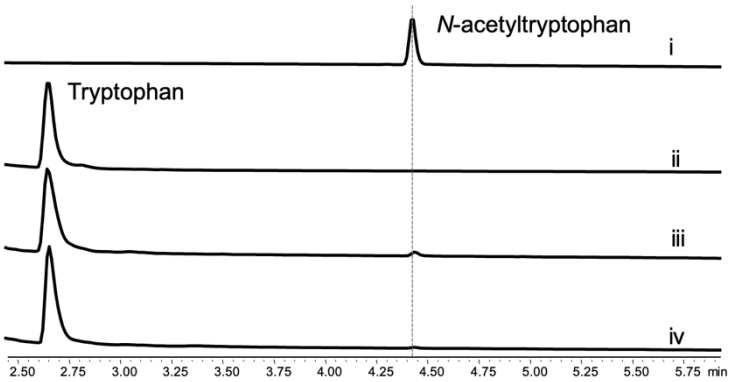


Fig. S3. In vitro characterization of IvoA acetyltransferase activity by HPLC.
The reaction condition is 100 μM of IvoA + 2 mM of ATP + 1 mM of L-tryptophan + 1 mM of AcCoA + 5 mM $MgCl_2$ in phosphate buffer (pH 7.5). Each trace represents: i) *N*-acetyltryptophan standard; ii) 30 min reaction; iii) 24 hrs reaction; iv) 24hrs reaction using boiled enzyme.

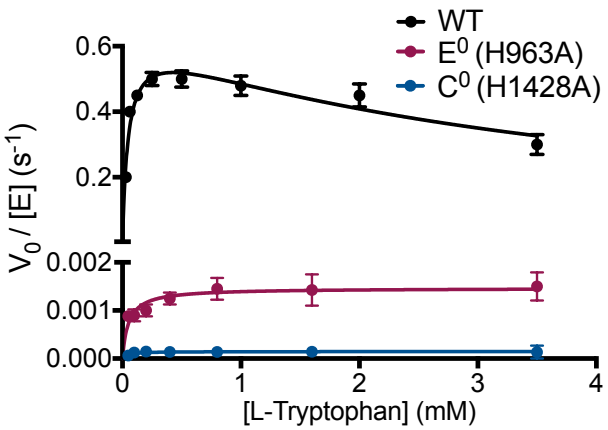


Fig. S4. Apparent steady-state kinetics of IvoA catalyzed stereoinversion.
Substrate inhibition was observed with WT enzyme. The kinetic constants were shown in the main text. For WT, 1 μM enzyme was used in each assay and the reaction was quenched after 2 min. For E^0 mutant, 10 μM enzyme was used in each assay and the reaction was quenched after 60 min. For C^0 mutant, 50 μM enzyme was used in each assay and the reaction was quenched after 60 min.

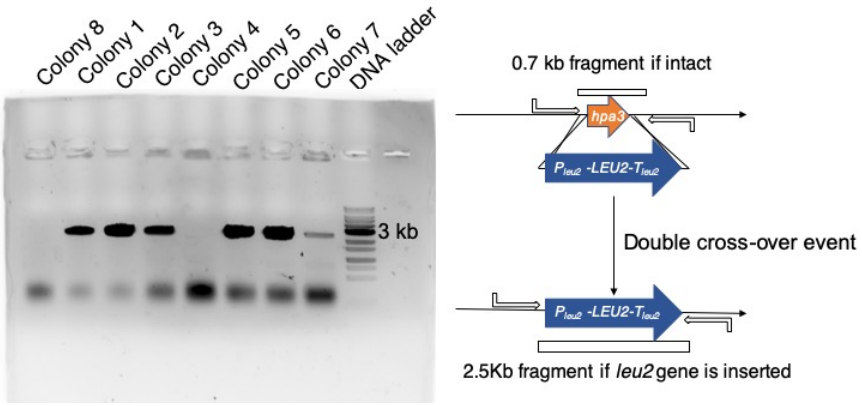


Fig. S5. Gene-knockout of *hpa3* in yeast.
Replacement of *hpa3* gene by LEU2 marker. Successful gene replacement will cause size change of PCR fragments. The integration was confirmed by colony PCR.

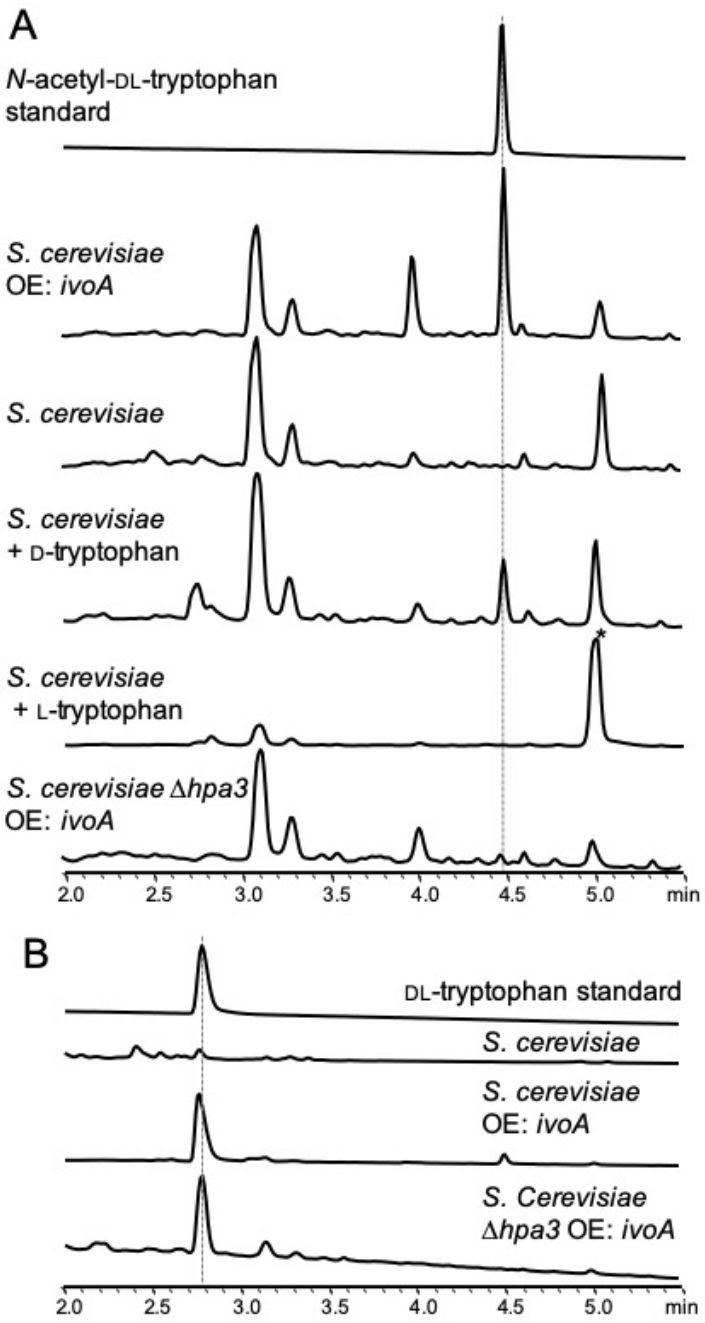


Fig. S6. Characterization of IvoA activity in vivo.

A) HPLC analysis of extracellular metabolites extracted from the culture medium. Excess L-tryptophan fed to the culture was converted to tryptophol (denoted by *) B) HPLC analysis of intracellular metabolites extracted from the yeast cell pellets.

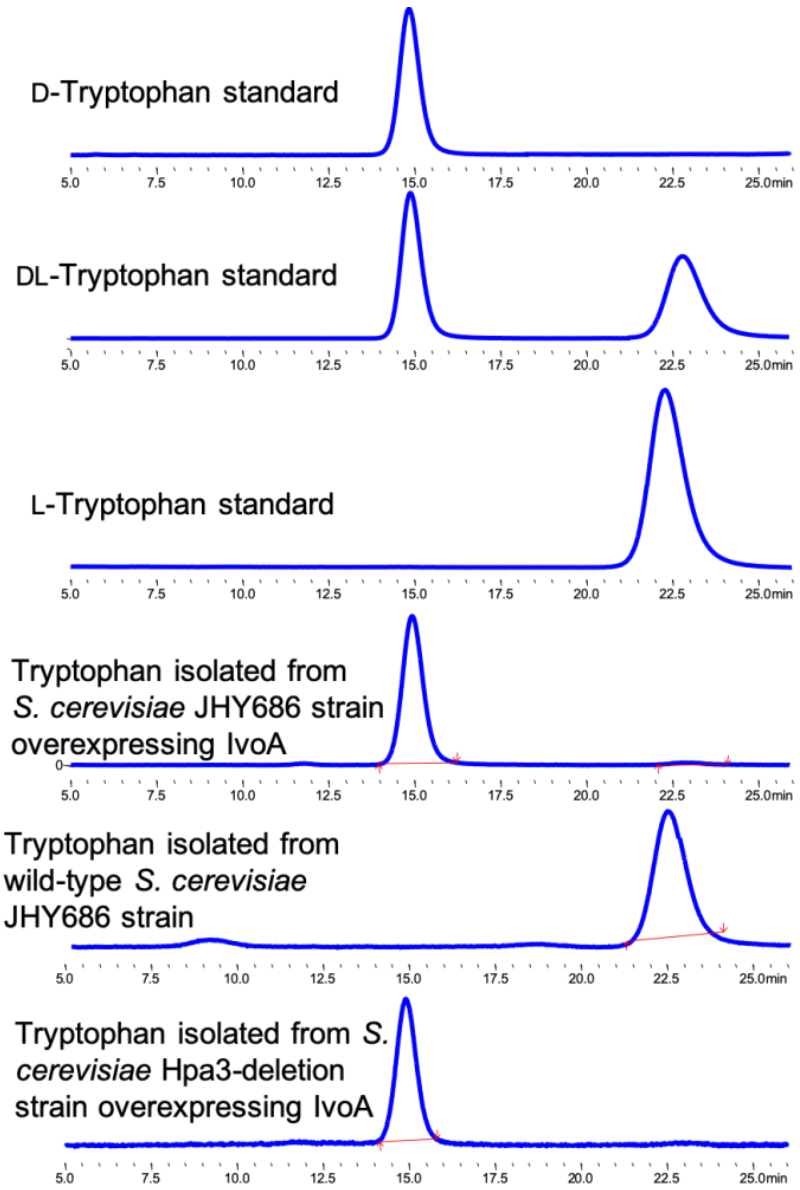


Fig. S7. Chiral-HPLC analysis of the purified tryptophan from yeast cells.
Note that D-enantiomer is eluted earlier than L-enantiomer**.** The analysis was performed by using a Crownpak® CR(+) column (150 mm x 4 mm x 3.5 μm, Daicel) at room temperature. The mobile phase was aq. $HClO_4$ 1% (w/v) supplemented with 15% (v/v) MeOH and the flow rate was 1.0 ml/min.

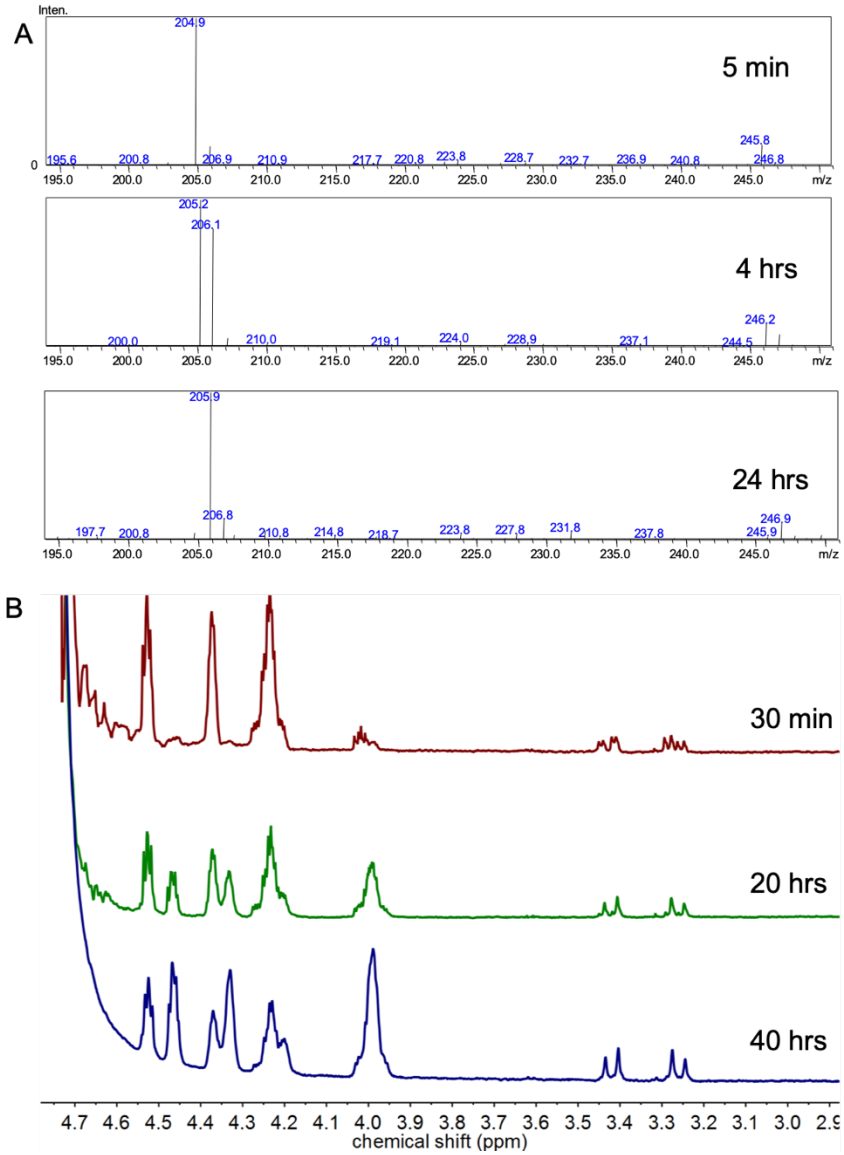


Fig. S8. In vitro hydrogen-deuterium exchange assay of IvoA with D-tryptophan.
A) Mass-spectrometry analysis shows the +1 Da mass shift of tryptophan in D_2O. B)^{1}H-NMR spectra indicate hydrogen-deuterium exchange took place at the α position. The observation is similar to that with L-tryptophan, which indicates that epimerization also occurs with D-tryptophan as substrate.

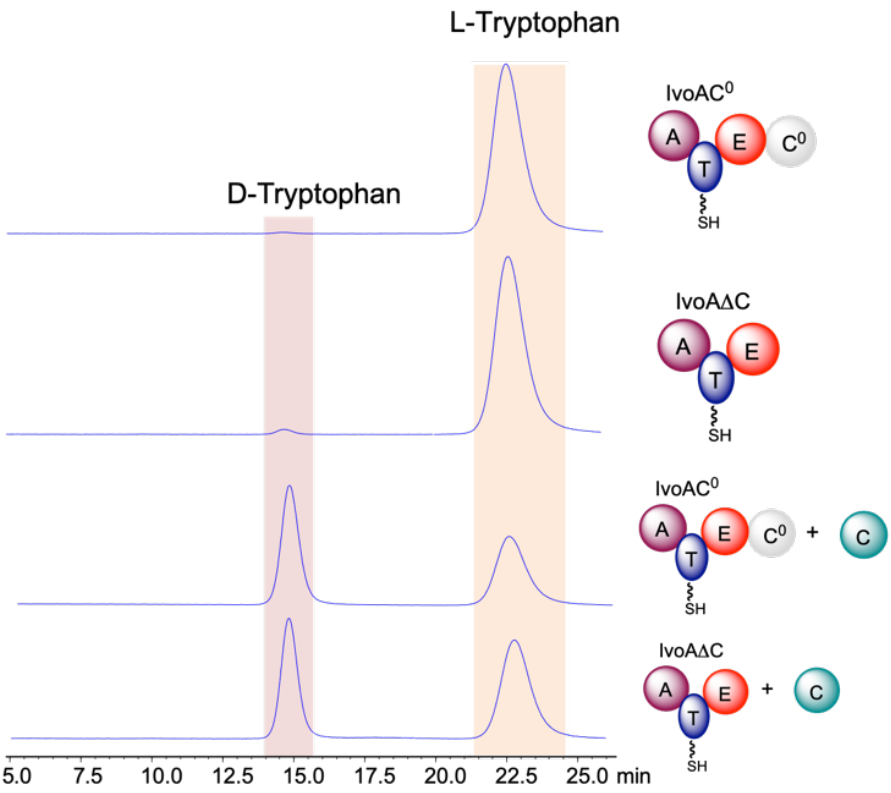


Fig. S9. Complementation of IvoA mutant with standalone IvoA-C.
Assay was performed by using 20 μM enzyme and 1 mM substrates. The impaired catalytic activity of C domain mutant or truncation variant can be complemented by adding standalone C domain in trans.

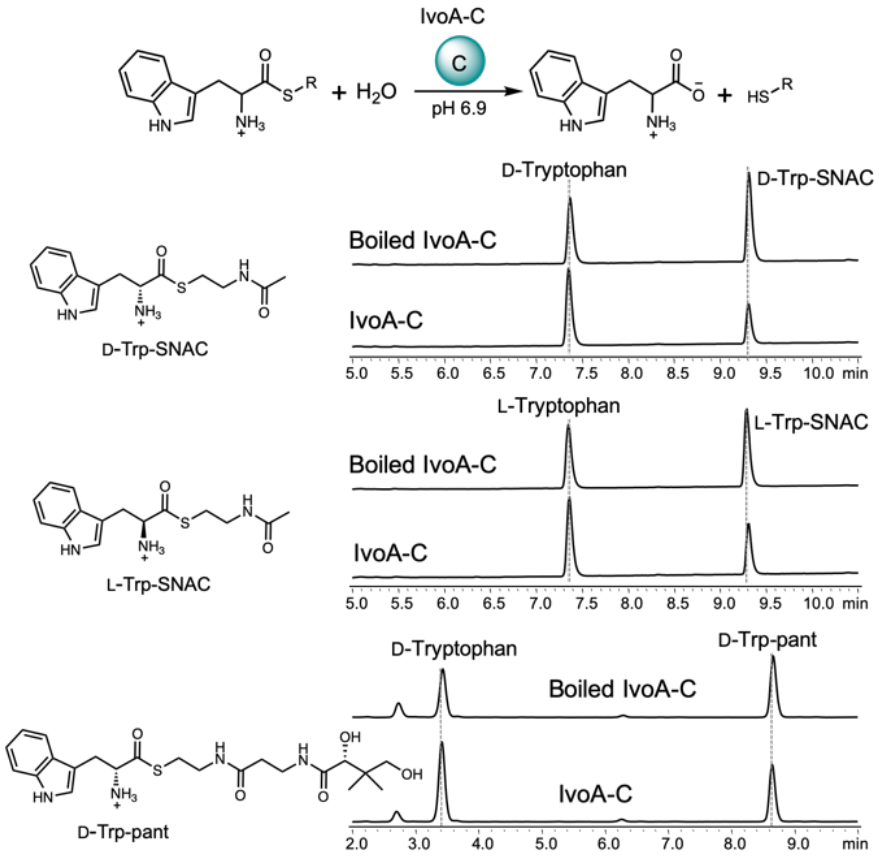


Fig. S10. In vitro assay of IvoA-C with synthetic thioester substrates.
Synthetic thioester substrates (DL-tryptophan-S-*N*-acetylcysteamine, DL-Trp-SNAC; D-tryptophan-S-pantatheine, D-Trp-pant) were incubated with standalone IvoA C domain at pH 6.9. Enzymes were boiled to measure the nonenzymatic hydrolysis. Free tryptophan and tryptophanyl thioesters were separated by HPLC. The enzyme catalyzed hydrolysis rate is not significantly different from noncatalyzed reaction, which indicates that these synthetic thioesters are not good substrate for IvoA-C.

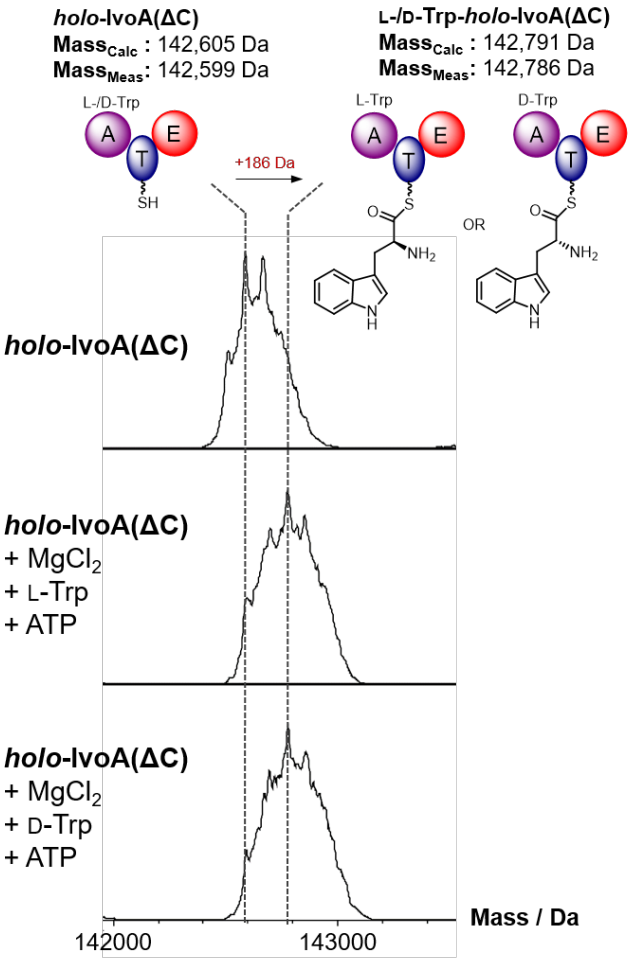


Fig. S11. Loading of D/L-tryptophan to IvoAΔC monitored by intact protein mass spectrometry.
Intact mass spectra of *holo*-IvoAΔC (top), and following incubation with $MgCl_2$, ATP and L-/D-tryptophan (middle and bottom). Loading reaction conducted with L- and D-tryptophan resulted in a +186 Da mass shift to the intact the intact *holo*-IvoAΔC protein.

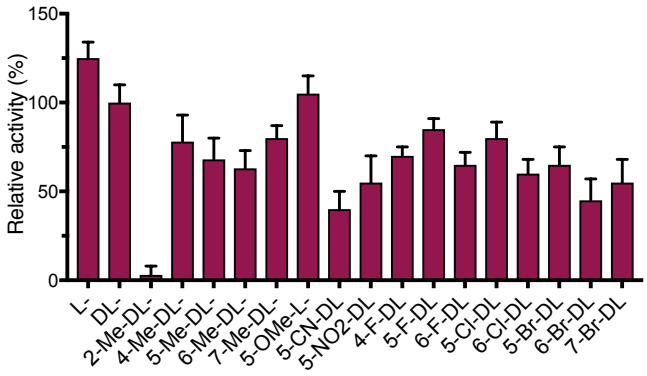


Fig. S12. Adenylation assay of IvoA A domain with substituted tryptophan amino acids.
The reaction was performed similarly according to the assay described in the caption of Fig. S2.

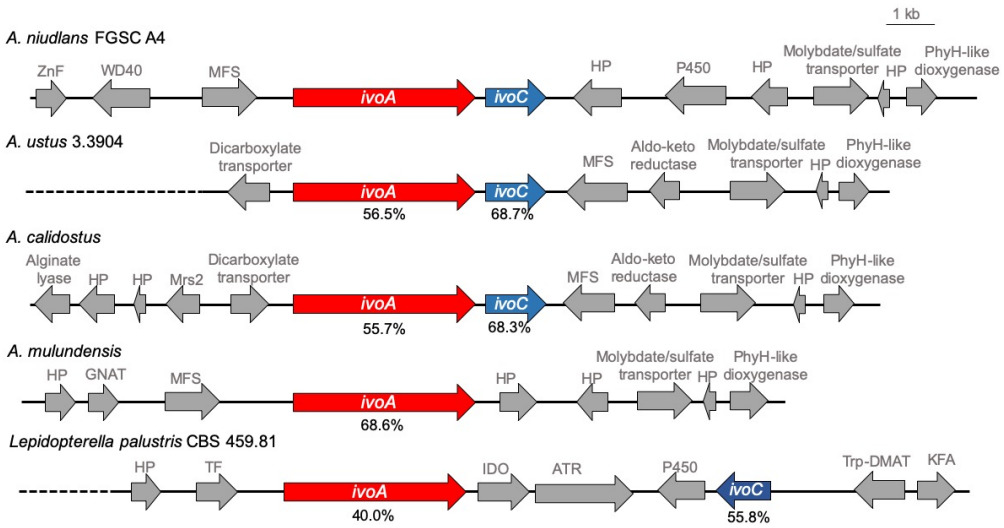


Fig. S13. Genomic context analysis of *ivoA* homologues found in other fungi.
Numbers below open reading frame indicate the amino acid sequence identity of IvoA and IvoC homologues to *A. nidulans* proteins. Abbreviations to follow: ZnF, zinc-finger protein; MFS, major-facilitator superfamily; HP, hypothetical protein; IDO, indoleamine 2,3-dioxygenase; ATR, NRPS-like carboxylic acid reductase harboring domain architecture as A-T-R; Trp-DMAT, dimethylallyl tryptophan synthase-like protein; KFA, kynurenine formyl amidohydrolase.

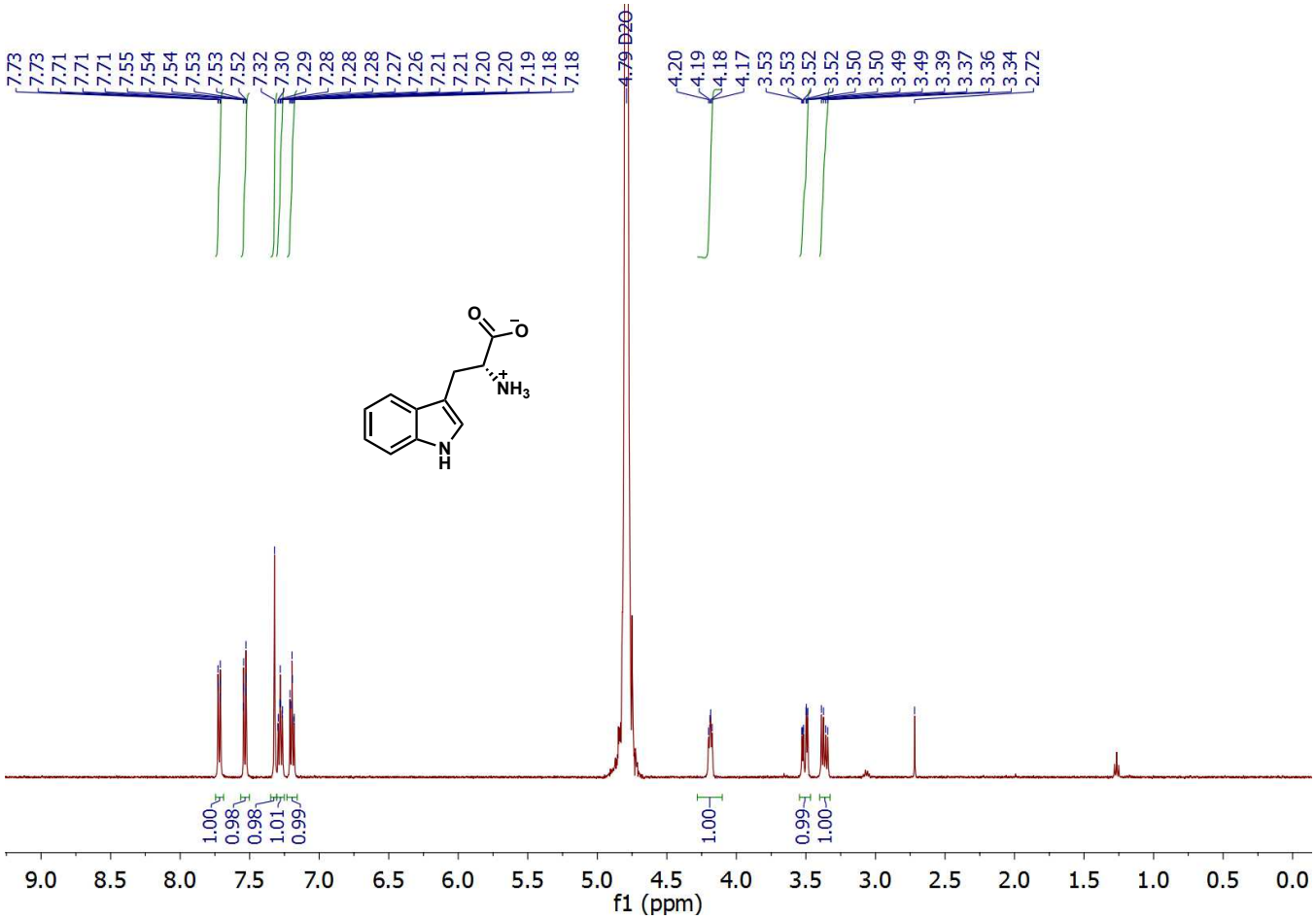


Fig. S14. ^{1}H-NMR spectrum (500 MHz) of D-tryptophan isolated from yeast overexpressing IvoA in D_2O.

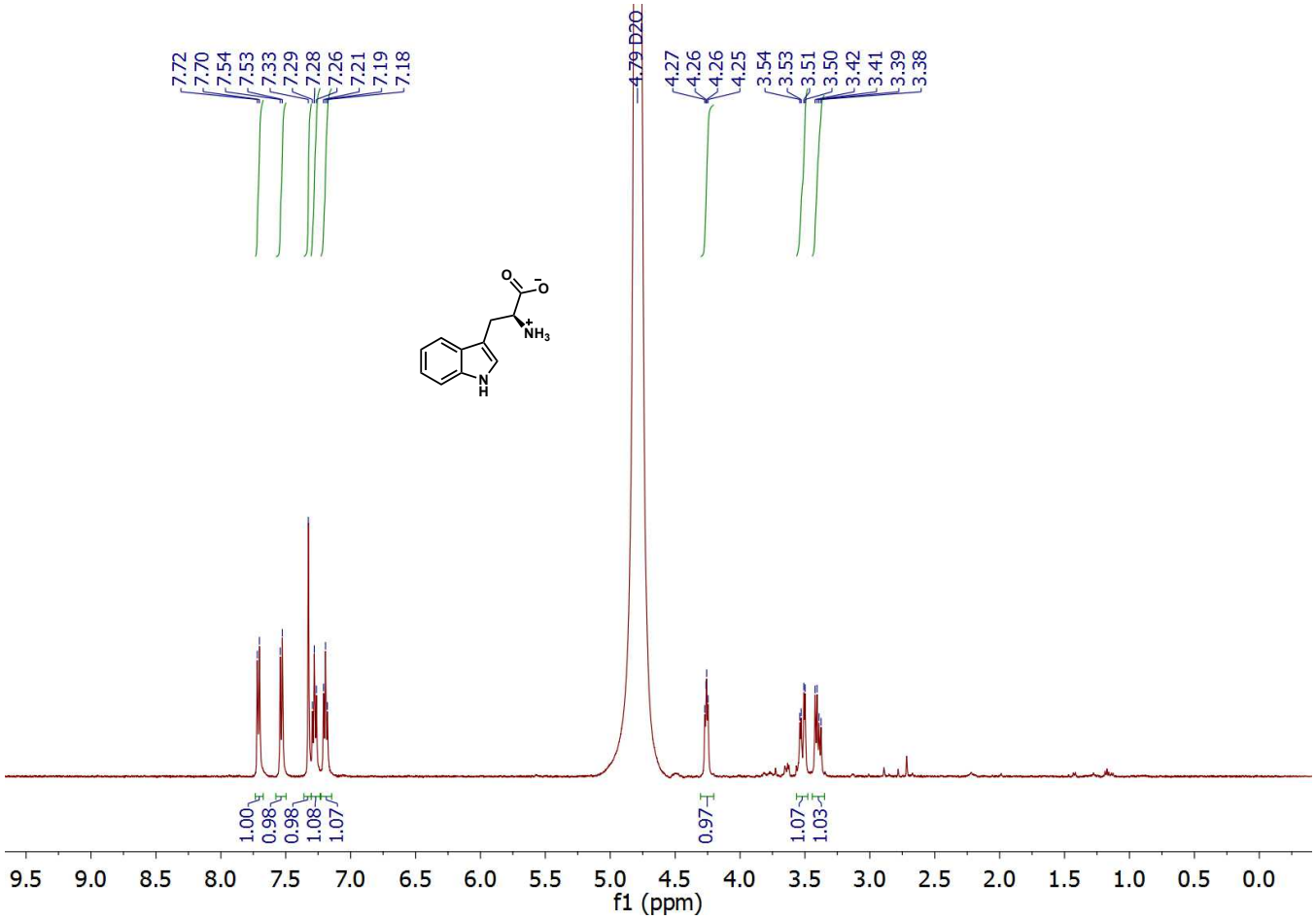


Fig. S15. ^{1}H-NMR spectrum (500 MHz) of L-tryptophan isolated from yeast in D_2O.

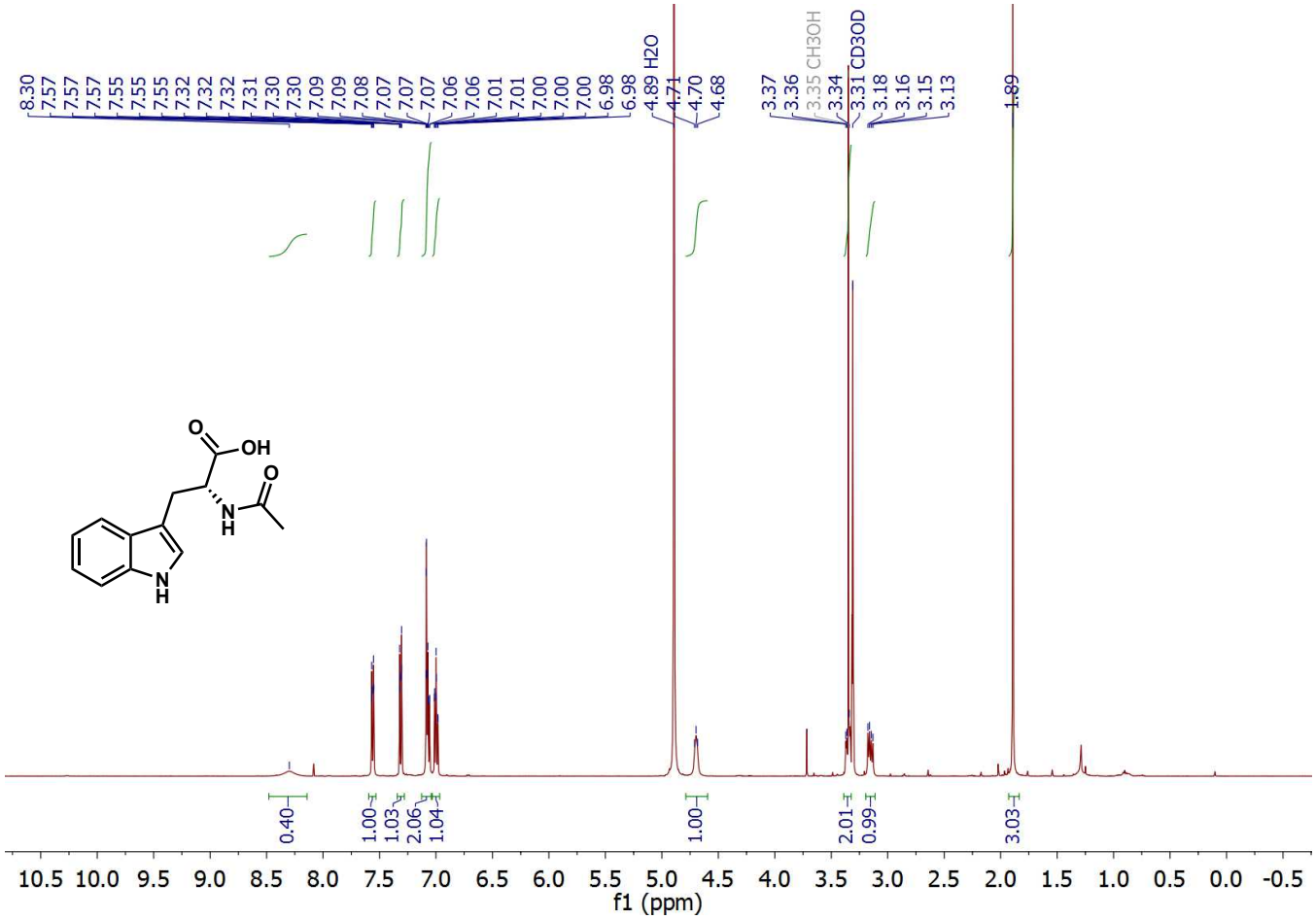


Fig. S16. ^{1}H-NMR spectrum (500 MHz) of isolated *N*-acetyl-D-tryptophan in MeOD.

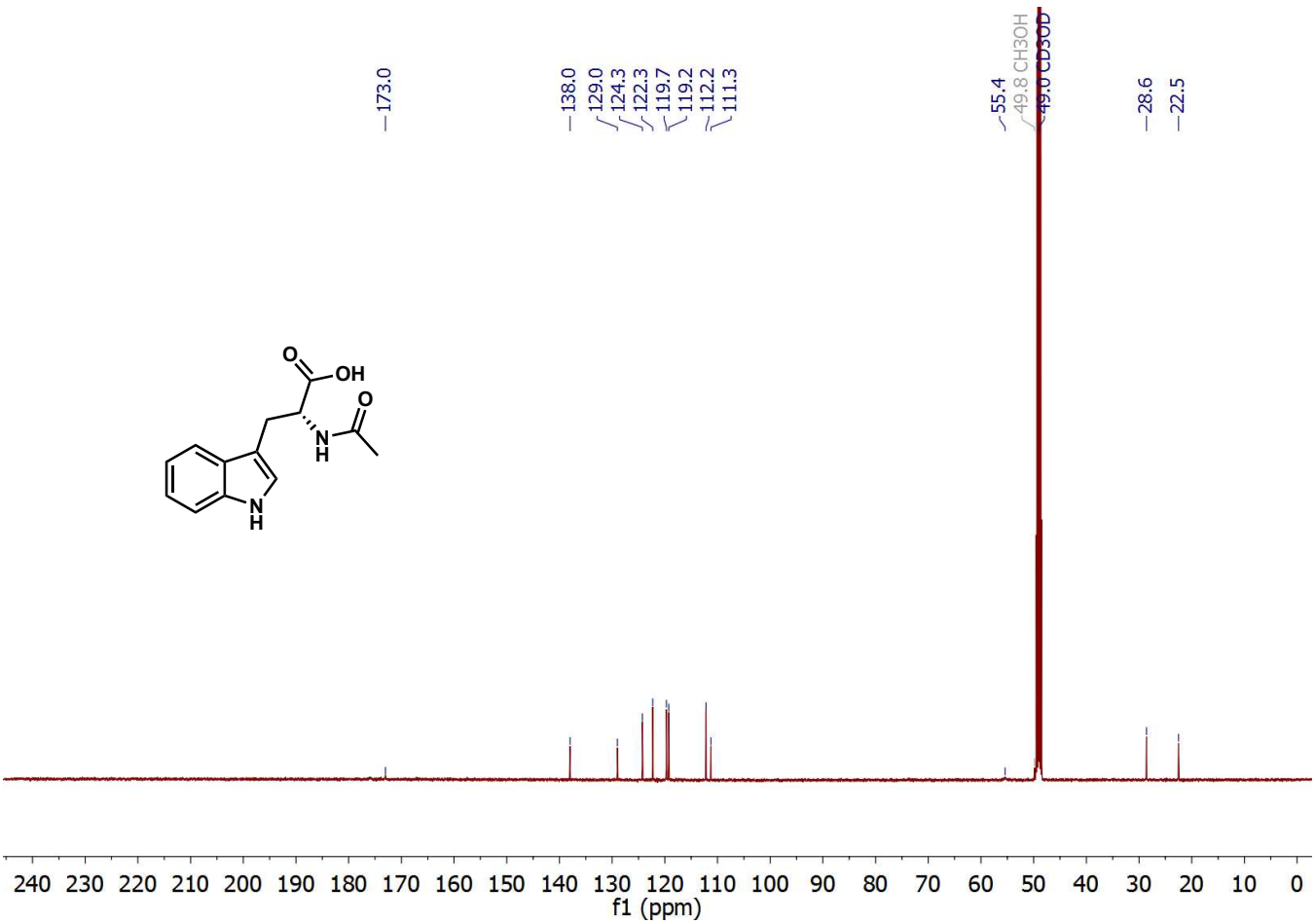


Fig. S17. ^{13}C-NMR spectrum (125 MHz) of isolated *N*-acetyl-D-tryptophan in MeOD.

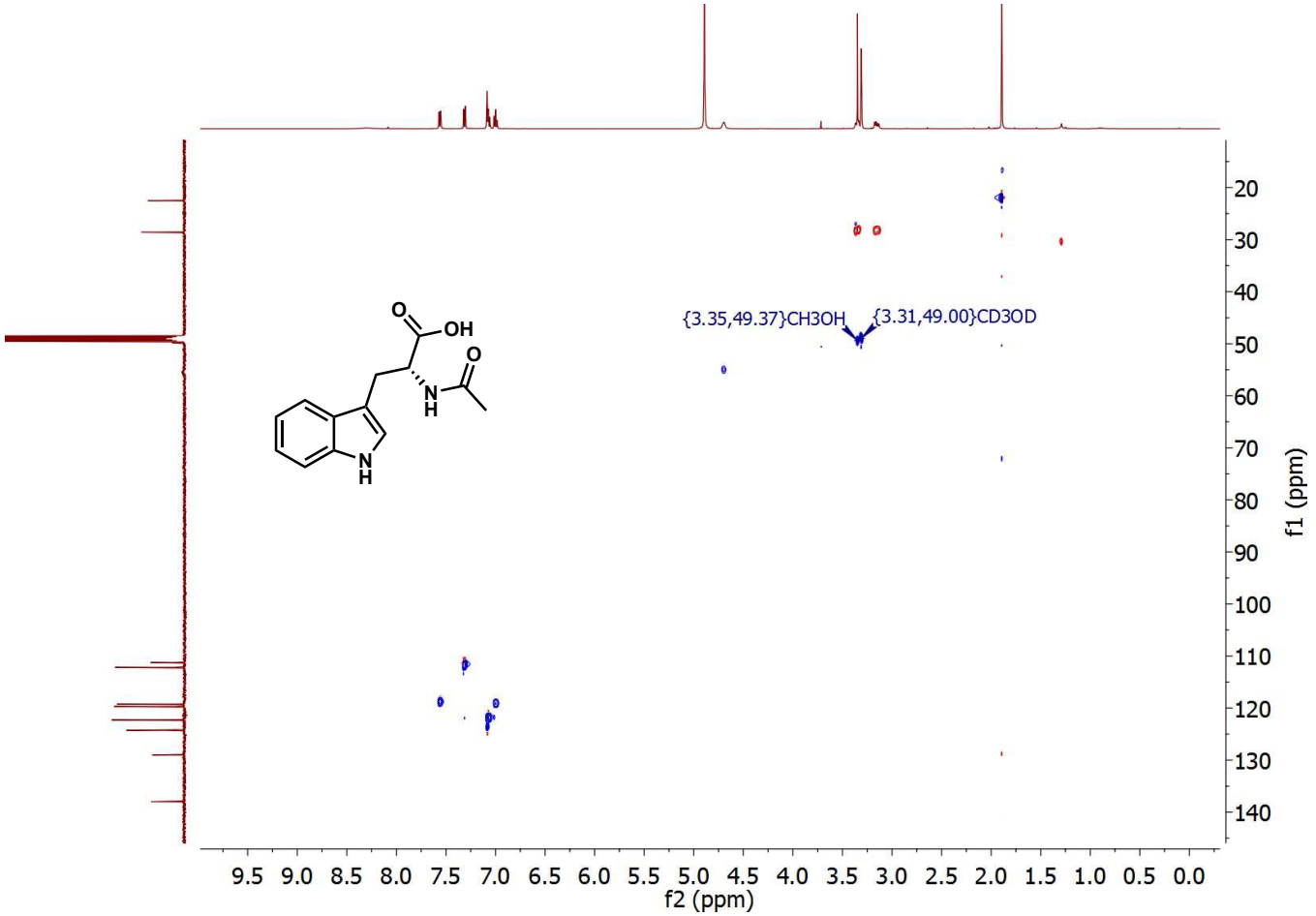


Fig. S18. HSQC NMR spectrum (500 MHz) of isolated *N*-acetyl-D-tryptophan in MeOD.

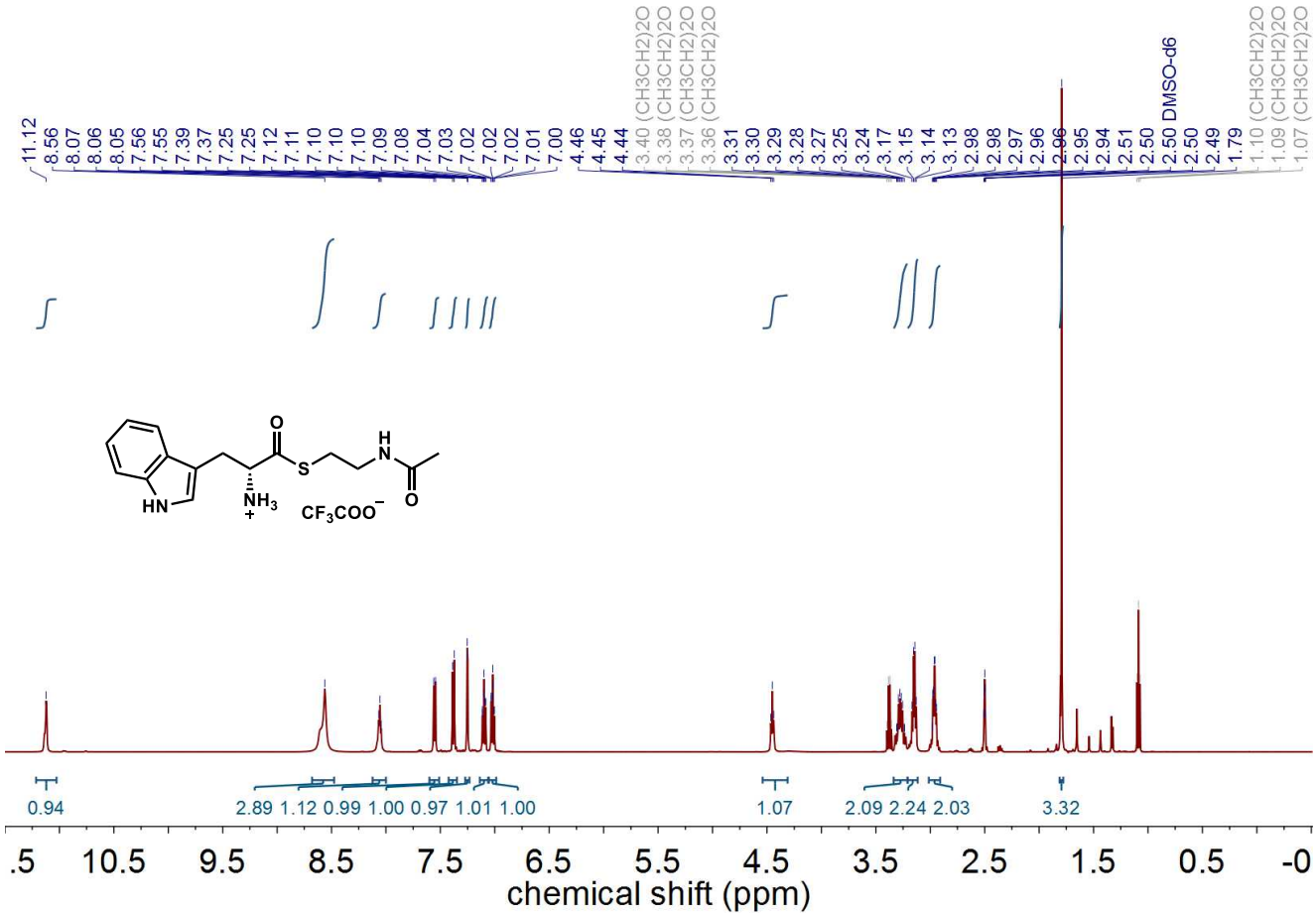


Fig. S19. ^{1}H-NMR spectrum (500 MHz) of synthesized D-Trp-SNAC in d_6-DMSO.

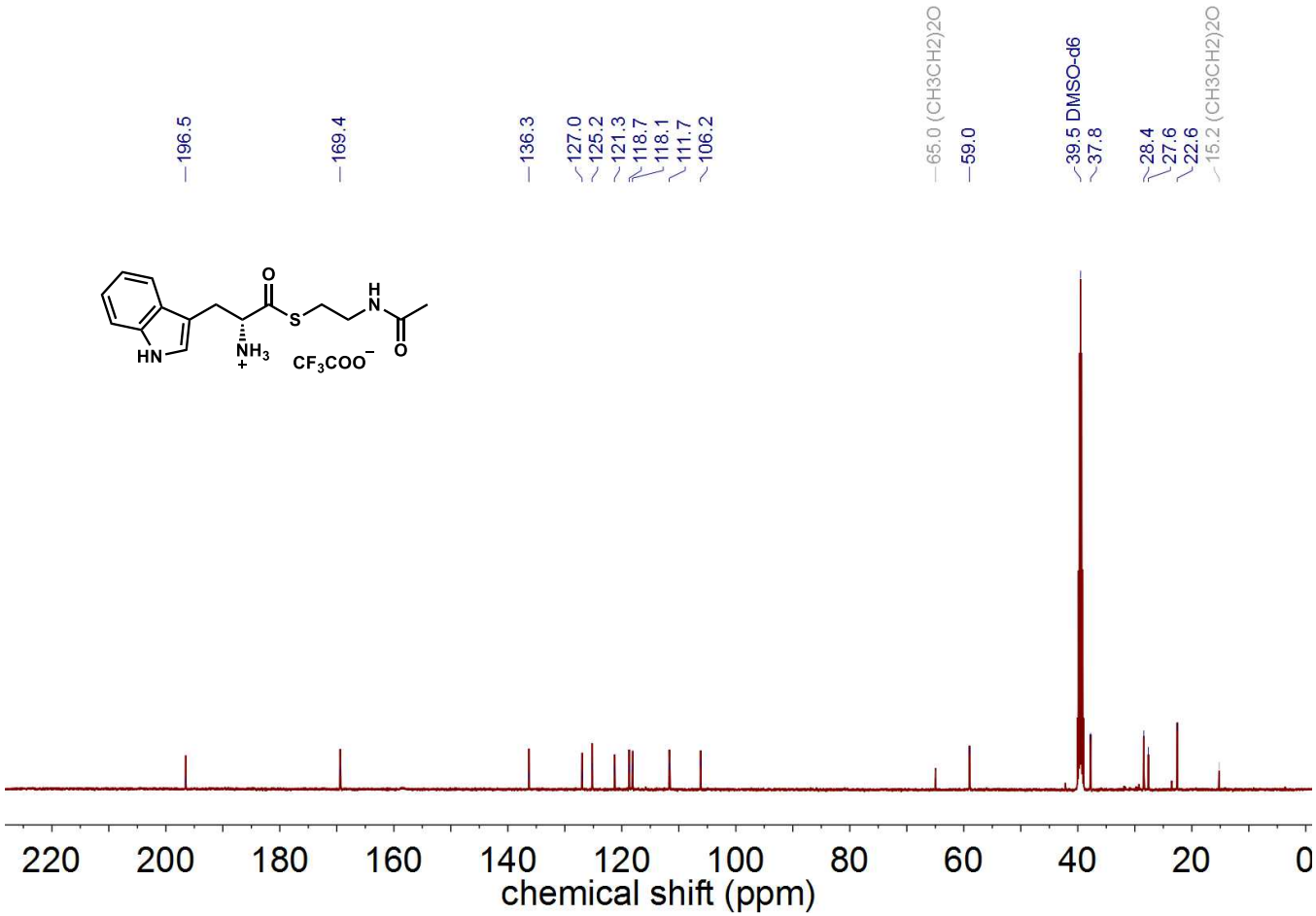


Fig. S20. ^{13}C-NMR spectrum (125 MHz) of synthesized D-Trp-SNAC in d_6-DMSO.

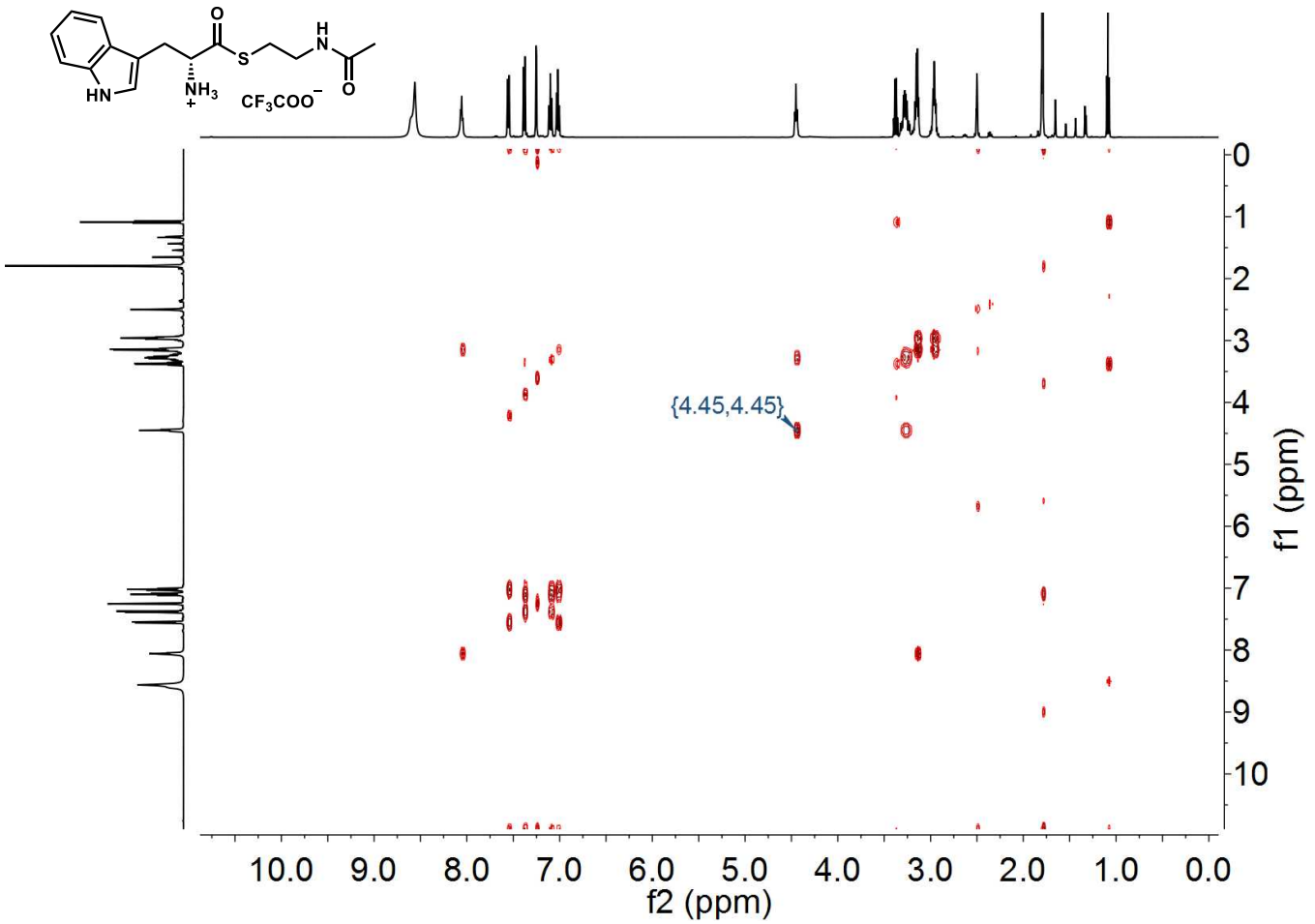


Fig. S21. COSY NMR spectrum (500 MHz) of synthesized D-Trp-SNAC in d_6-DMSO.

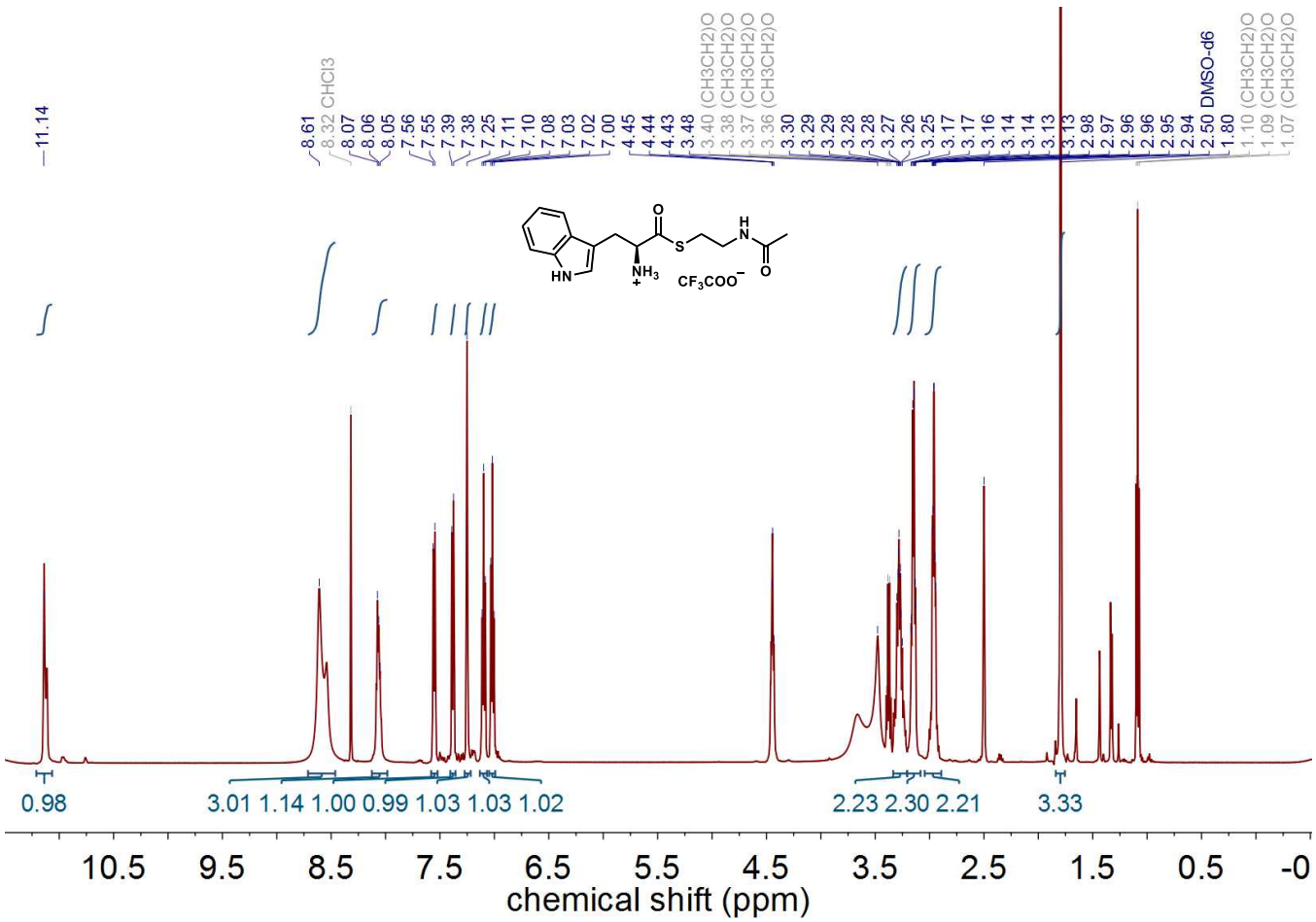


Fig. S22. ^{1}H-NMR spectrum (500 MHz) of synthesized L-Trp-SNAC in d_6-DMSO.

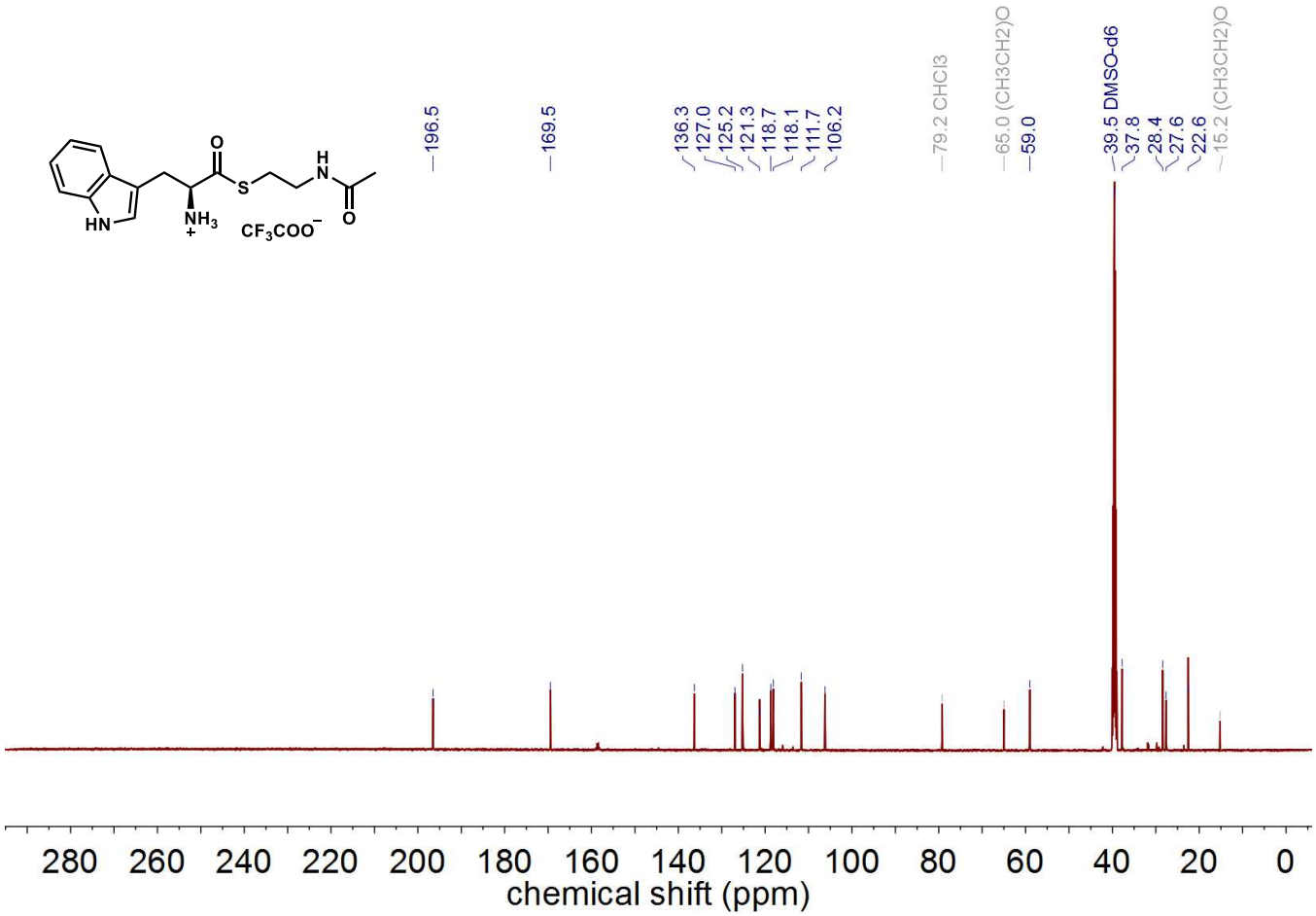


Fig. S23. ^{13}C-NMR spectrum (125 MHz) of synthesized L-Trp-SNAC in d_6-DMSO.

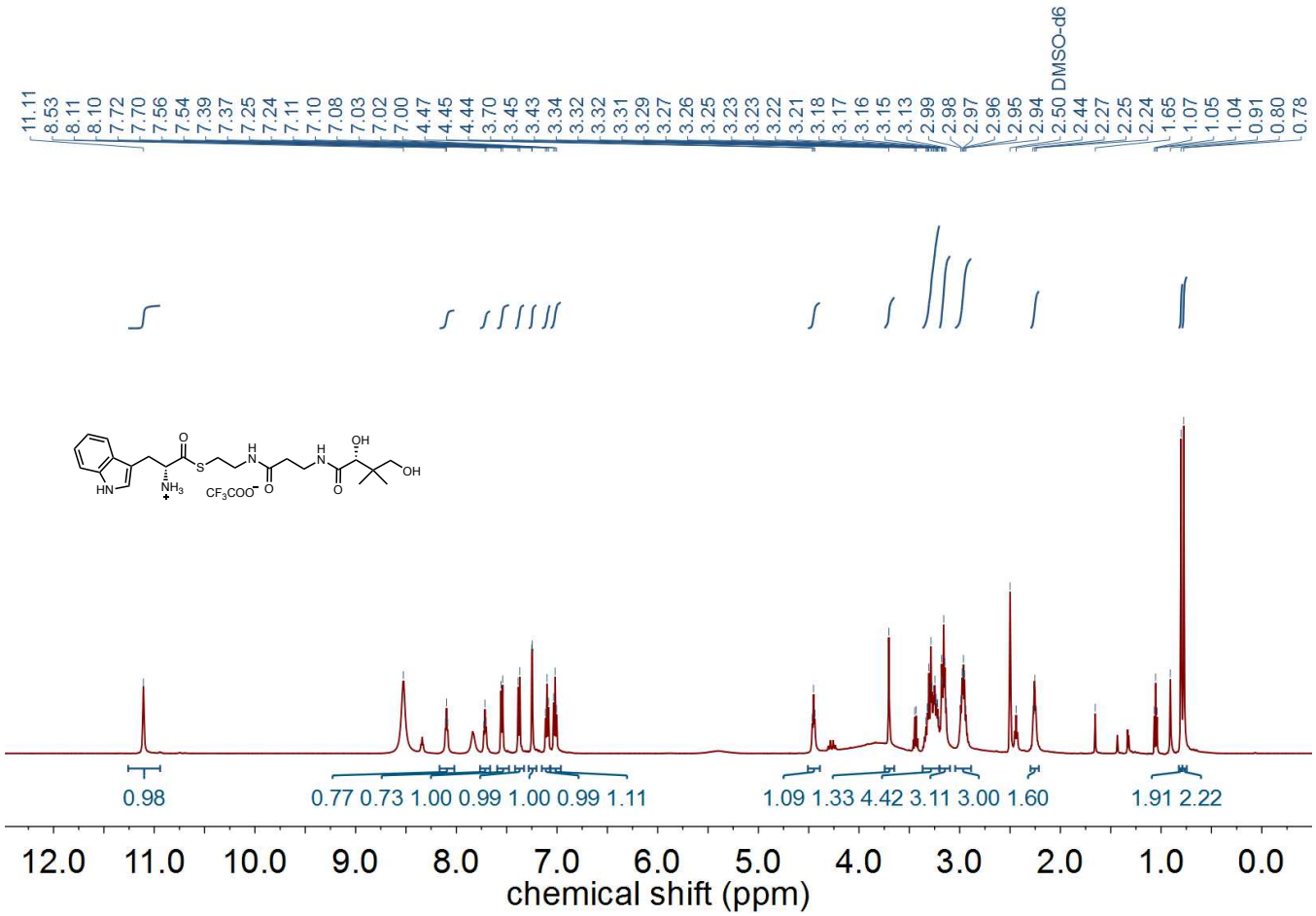


Fig. S24. ^{1}H-NMR spectrum (500 MHz) of synthesized D-Trp-pant in d_6-DMSO.

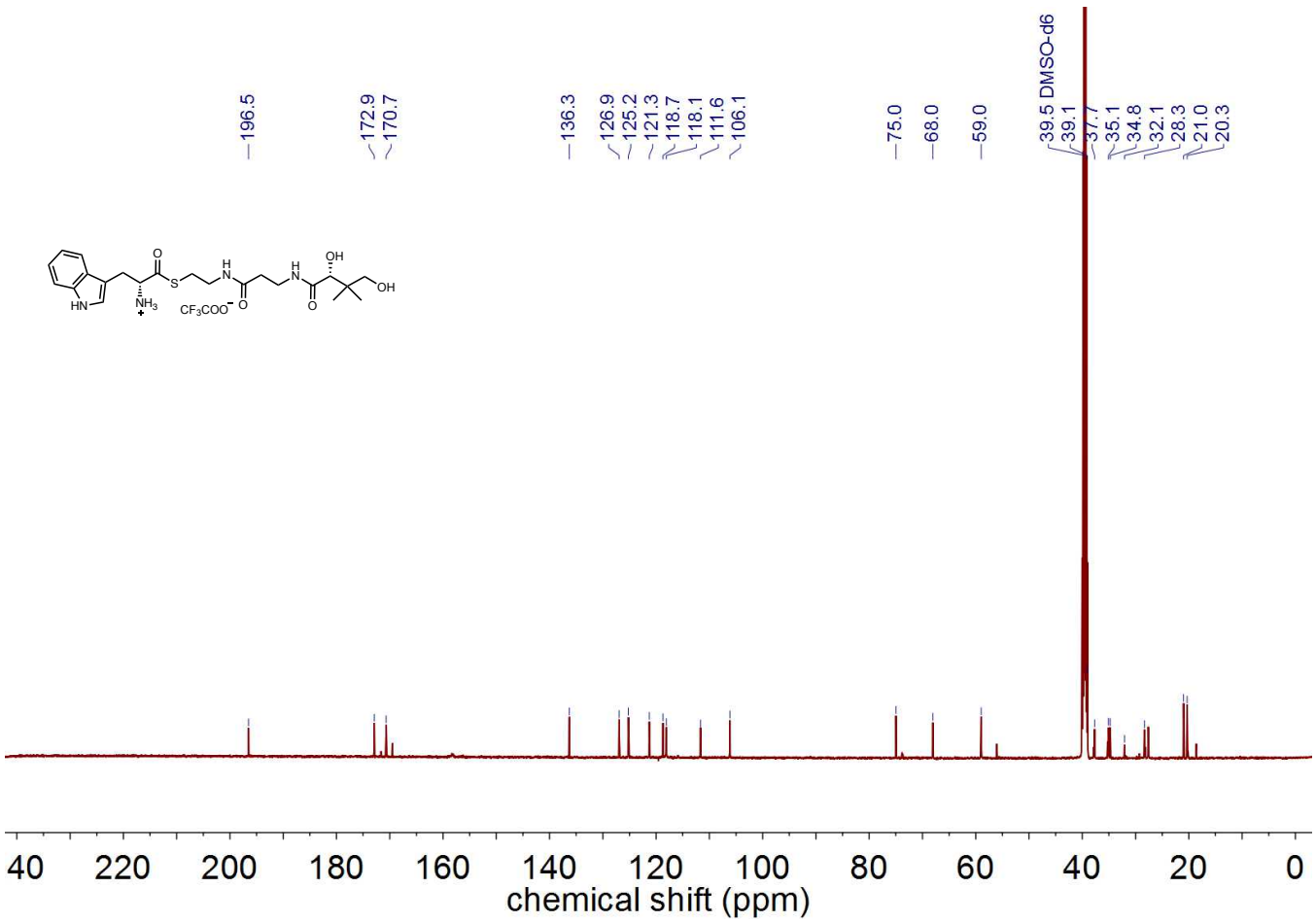


Fig. S25. ^{13}C-NMR spectrum (125 MHz) of synthesized D-Trp-pant in d_6-DMSO.

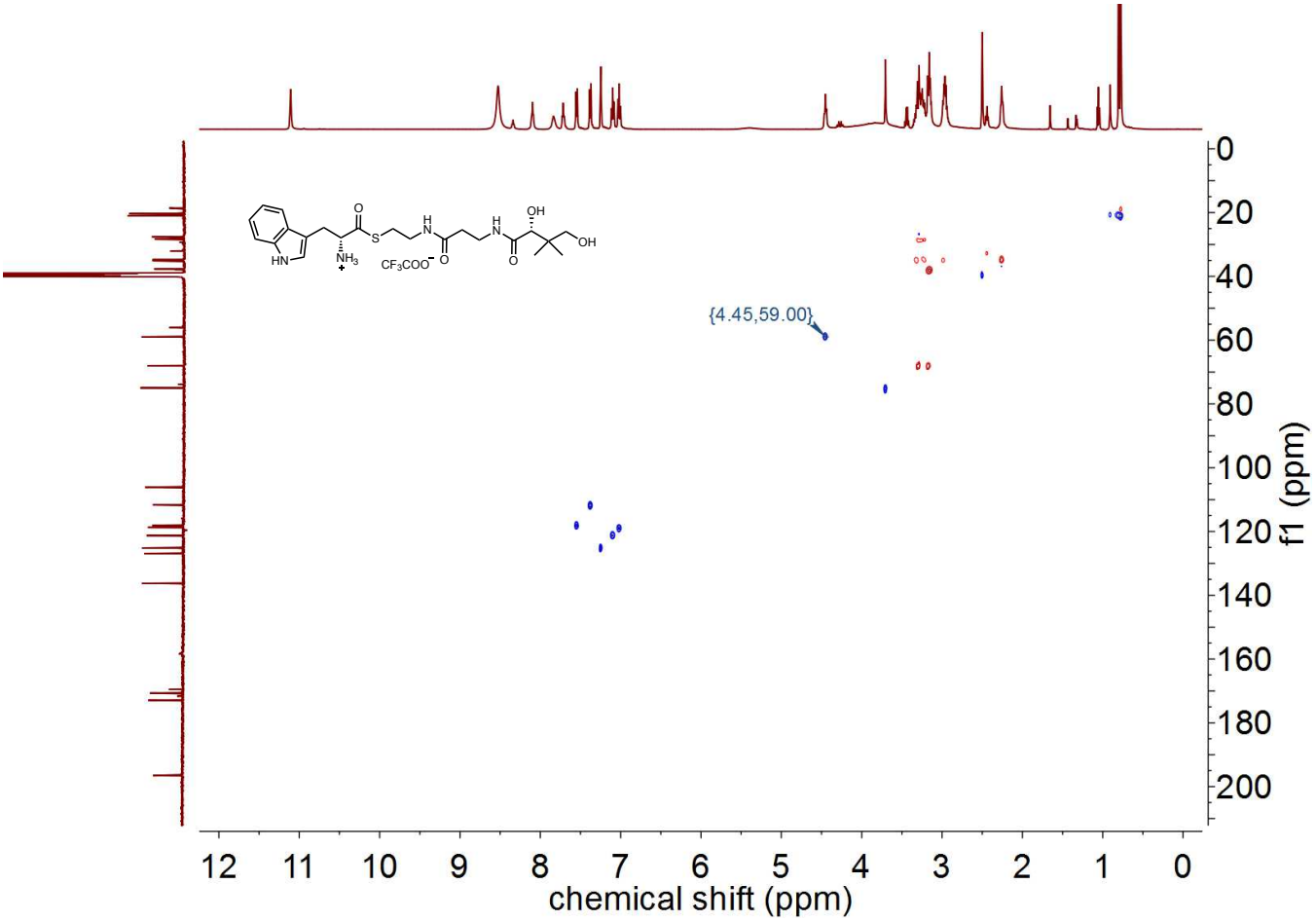


Fig. S26. HSQC NMR spectrum (500 MHz) of synthesized D-Trp-pant in d_6-DMSO.

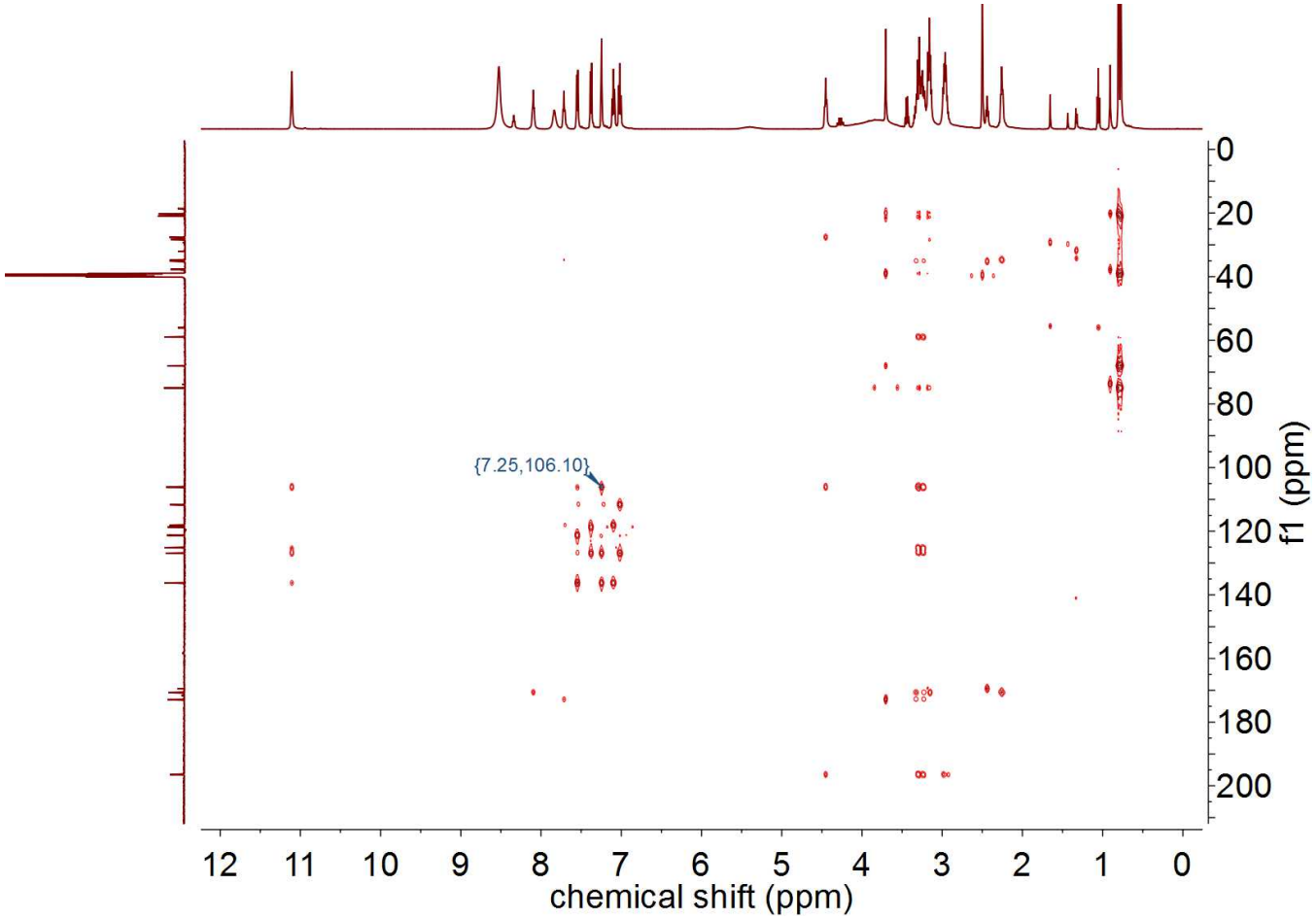


Fig. S27. HMBC NMR spectrum (500 MHz) of synthesized D-Trp-pant in d_6-DMSO.

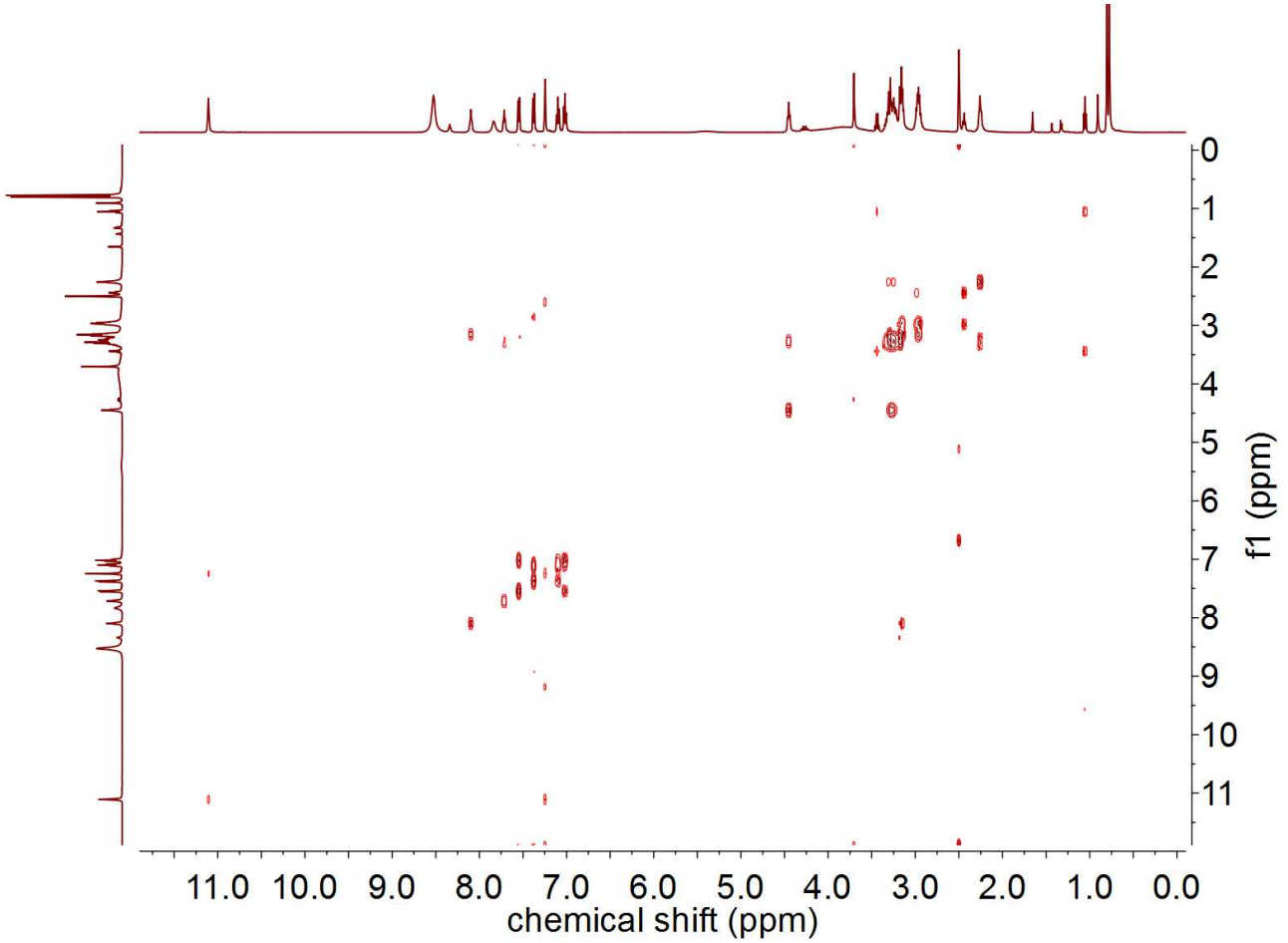


Fig. S28. COSY NMR spectrum (500 MHz) of synthesized D-Trp-pant in d_6-DMSO.

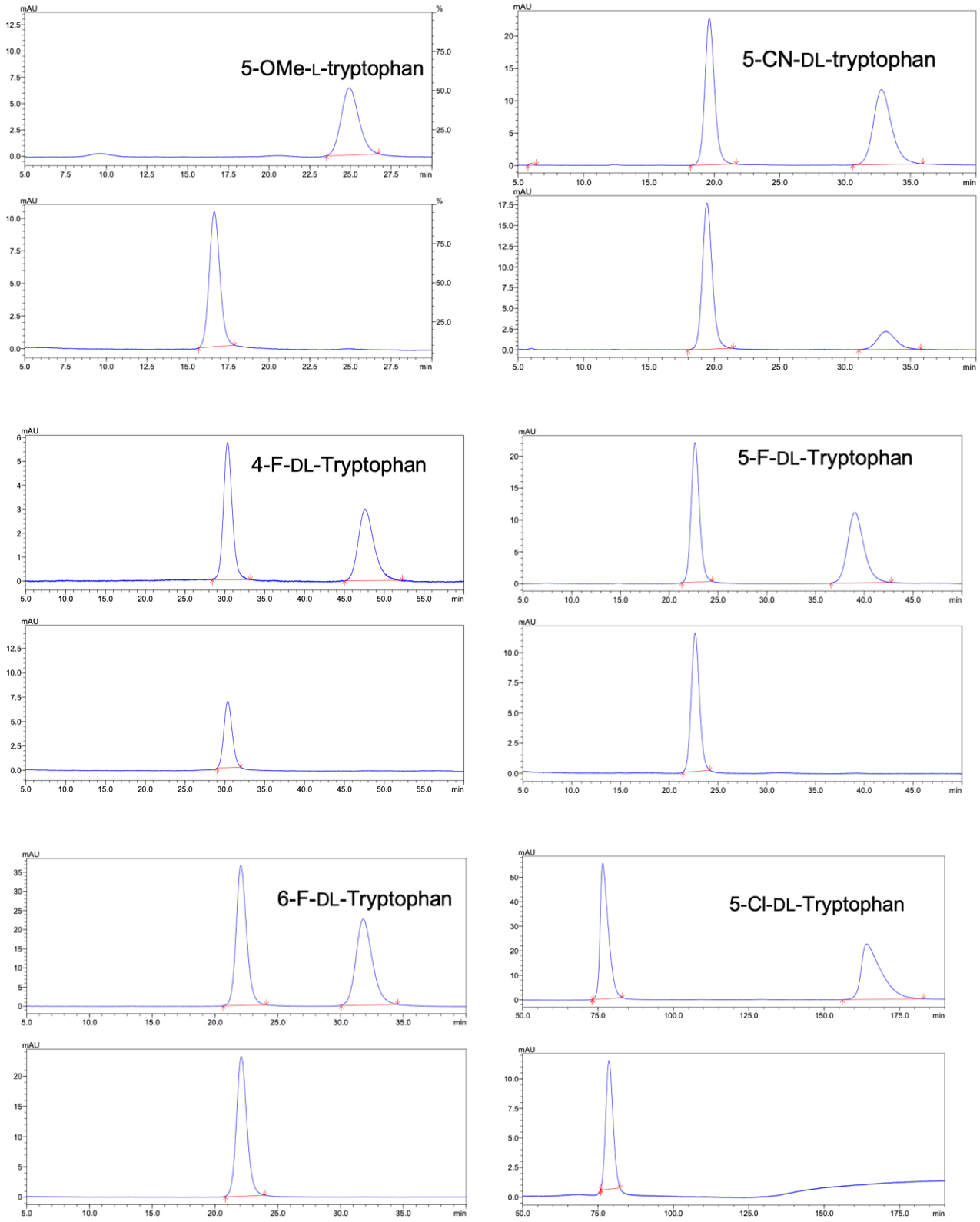


Fig. S29. Representative chiral HPLC traces (part I).
In each figure, the upper pannel shows the unreacted substrate, the bottom pannel shows the rection mixture after the stereoinversion. Note that the D-isomer is always eluted earlier than L-isomer.

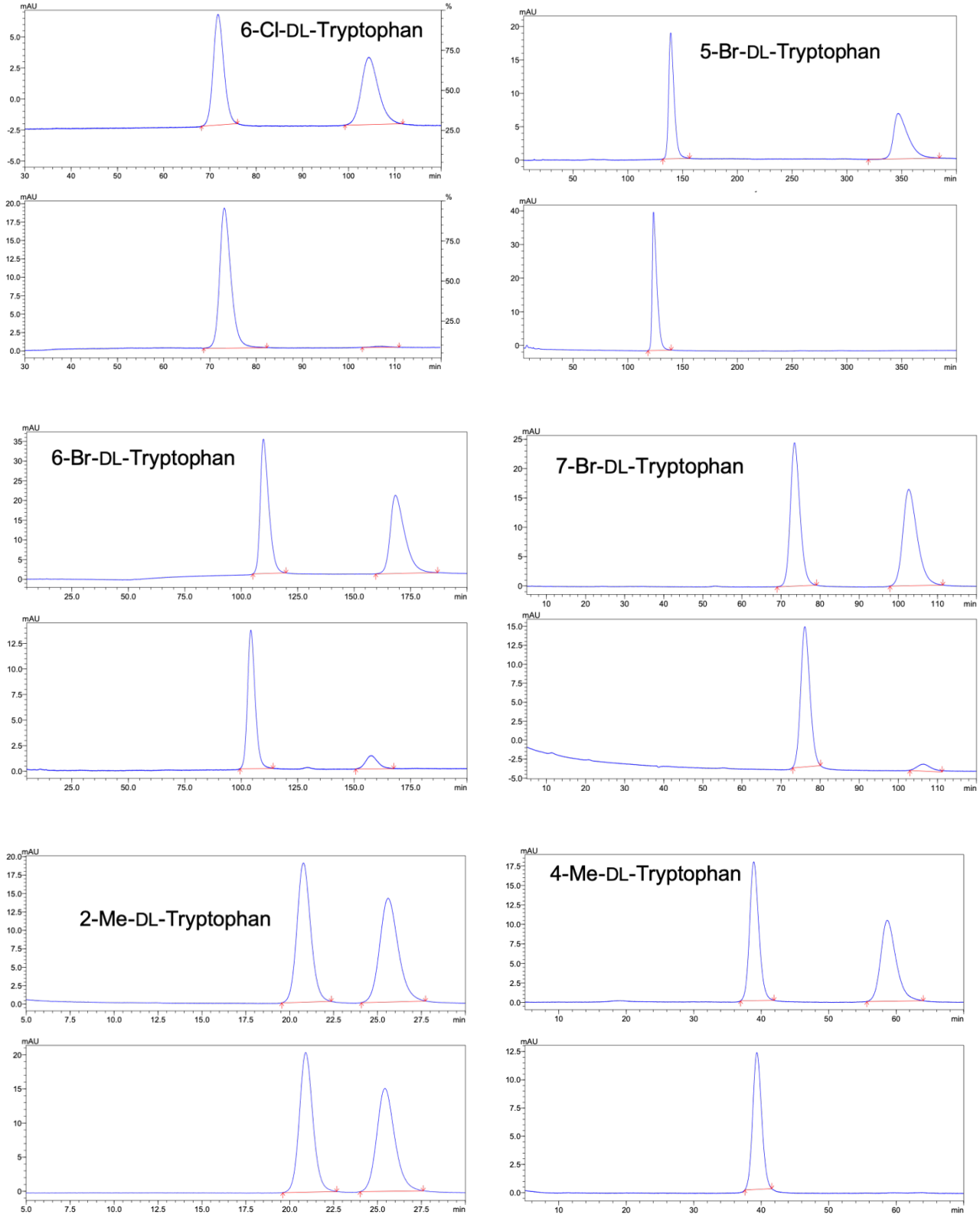


Fig. S30. Representative chiral HPLC traces (part II).
In each figure, the upper pannel shows the unreacted substrate, the bottom pannel shows the rection mixture after the stereoinversion. Note that the D-isomer is always eluted earlier than L-isomer.

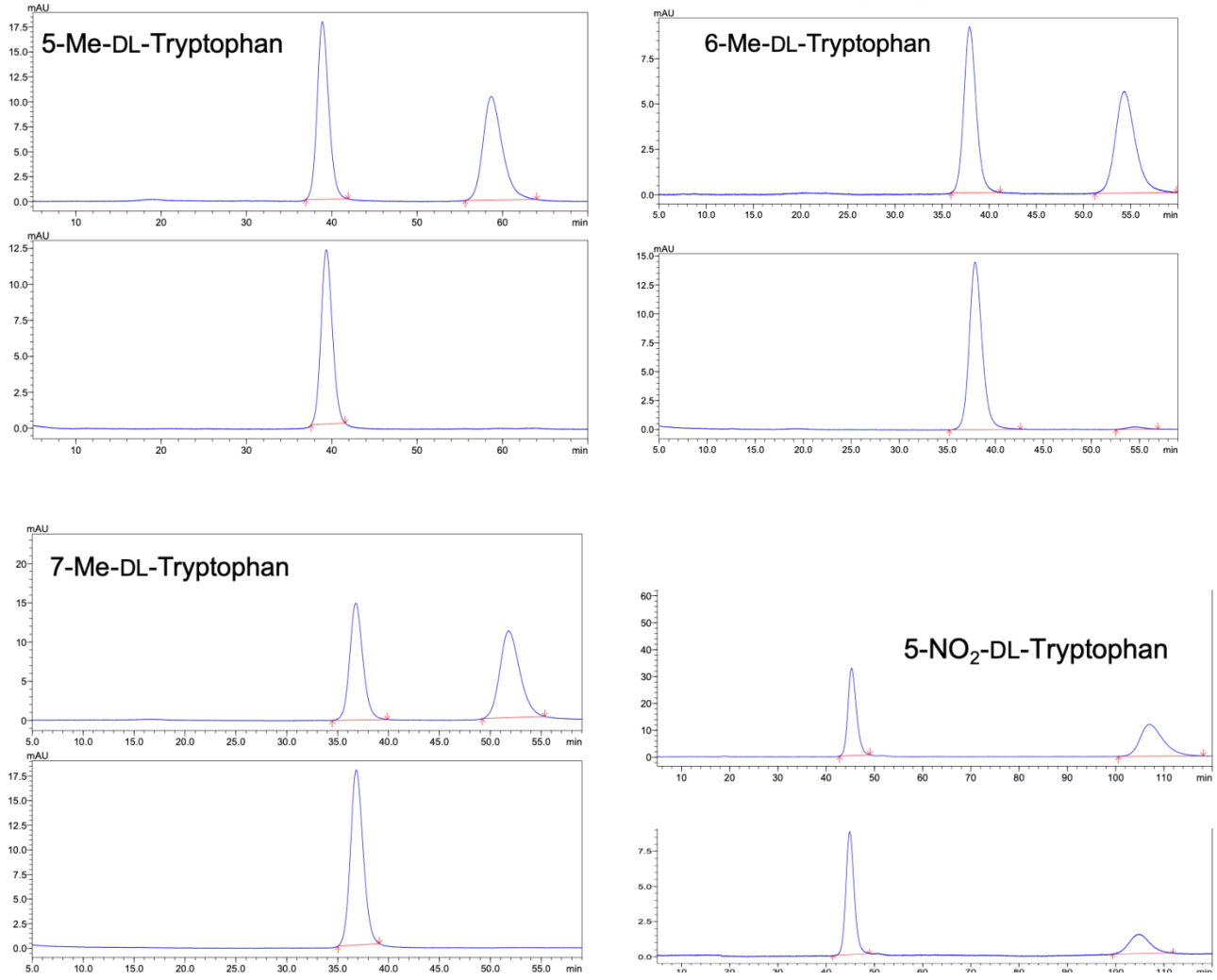


Fig. S31. Representative chiral HPLC traces (part III).
In each figure, the upper pannel shows the unreacted substrate, the bottom pannel shows the rection mixture after the stereoinversion. Note that the D-isomer is always eluted earlier than L-isomer.

4. References:

1. Liu, N.; Hung, Y.-S.; Gao, S.-S.; Hang, L.; Zou, Y.; Chooi, Y.-H.; Tang, Y. Identification and heterologous production of a benzoyl-primed tricarboxylic acid polyketide intermediate from the zaragozic acid a biosynthetic pathway. *Org. Lett.* 2017 19, 3560-3563
2. Kadi, N.; Challis, G. L. Chapter 17. Siderophore biosynthesis a substrate specificity assay for nonribosomal peptide synthetase-independent siderophore synthetases involving trapping of acyl-adenylate intermediates with hydroxylamine. *Methods Enzymol.* 2009, 458, 431-457.
3. Agarwal, V.; Diethelm, S.; Ray, L.; Garg, N.; Awakawa, T.; Dorrestein, P. C.; Moore, B. S. Chemoenzymatic synthesis of acyl coenzyme A substrates enables *in situ* labeling of small molecules and proteins. *Org. Lett.* 2015, 17, 4452-4455.