Support information for

Cyclodesulfurization Reaction Catalyzed by Artificial Metalloenzymes Containing Cobalt Protoporphyrin IX Cofactors under Green Aqueous Solvent Conditions

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General information

All the chemicals and reagents were purchased from commercial suppliers (SigmaAldrich, Bide Pharmatech, Aladdin, Energy Chemical, TCI) and used without any further purification, unless otherwise stated. *E. coli* strain Nissle 1917 was obtained from the *E. coli* Genetic Stock Center (CGSC) at Yale University. Ni-NTA Superflow resin was obtained from Solarbio. *E. coli* BL21(DE3) Competent Cell, Spin Miniprep, and Gel Extraction Kits were all obtained from Tiangen. Co(ppIX) was synthesized according to a previously reported procedure[1]. All reactions were carried out in oven-dried glassware with magnetic stirring. Silica gel chromatography purifications were carried out using AMD Silica Gel 60 230-400 mesh. Thin Layer Chromatography (TLC) and preparative TLC were carried out using Merck Millipore TLC silica gel 60 F254 glass plates. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a 400 MHz spectrometer in DMSO-*d6*. Chemical shifts for protons are reported in parts per million downfield from tetramethylsilane (TMS) and are referenced to residual protium in the NMR solvent (DMSO-*d6* = δ 2.62 ppm). NMR data are presented as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant in Hertz (Hz), integration. The experiments were performed in triplicate, and all data were obtained based on the average values.

Experimental procedures

Growth Media.

Luria-Bertani media was prepared as follows. For 1 L Luria-Bertani media, deionized H_2O was added with 10 g of peptone 140 (pancreatic digest of casein), 10 g of NaCl, 5 g of yeast extract autolyzed low sodium. Luria-Bertani agar plates were prepared by adding 15 g agar to 1 L Luria-Bertani media with ampicillin antibiotic selection. Ampicillin was added to media and plates to a final concentration of 100 mg/L.

General Methods for Expression of *Vitreoscilla* Hemoglobin.

The VHb gene and promoter region (promoter-VHb) were amplified by polymerase chain reaction (PCR) with Pfu polymerase and a pair of primers (the forward primer 5′-CCCAAGCTTACAGGACGCTGGGGAAAGT-3′; the reverse primer 5′-CCGGAATTCTTAATGATGATGATGATGATGTTCAACCGCTTGAGCGTACAAATCT-3′). The promoter-VHb fragment was excised from the plasmid DNA through restriction enzyme digestion with EcoRI and HindIII. Finally, the promoter-VHb fragment was cloned into the pUC-19 plasmid and the recombinant plasmid was transformed into *E. coli* BL21 (DE3) cells for protein expression. All VHb proteins were engineered to contain a C-terminal polyhistidine tag for subsequent purification. BL21(DE3) competent cells were used for expression of normal VHb and variants with natural hemin cofactor. The related primers of the VHb mutant are from the enzyme library of our group. Competent cells were transformed with the PUC-19 vector encoding the appropriate VHb variant and selected on Luria-Bertani agar plates containing ampicillin (100 mg/L) for strain selection. Single colonies were inoculated into 5 mL of Luria-Bertani media containing ampicillin (100 mg/L), and incubated at 37°C with shaking (180 rpm) for 10 to 15 hours. The overnight cultures were then transferred to 1 L of Luria-Bertani media with ampicillin, and grown at 37°C until the optical density at 600 nm (OD_{600}) reached 1.5. Induction of VHb expression was

initiated by shifting the culture to an anaerobic environment, followed by incubation at 25°C with shaking at 110 rpm for 30 hours. Cell cultures were harvested by centrifugation at 5000 rpm. The overall pelleted bacteria were dissolved in 50 mL of 20 mM phosphate buffer (pH = 7.4). After sonication for 30 min on ice, the cell lysates were centrifuged at 12,000 rpm for 30 min to clarify the supernatant, which was subsequently used for protein purification.

Expression-based Porphyrin Substitution of *Vitreoscilla* Hemoglobin Containing Artificial Metalloporphyrins.

The expression-based porphyrin substitution for generating *Vitreoscilla* hemoglobin (VHb) containing artificial metalloporphyrins was performed using *E. coli* strain Nissle 1917, which harbors chromosomal copies encoding the heme acquisition system "*chu*", which have been demonstrated to enhance heme incorporation during hemoglobin expression times[2]. Thus, Nissle 1917 functions as a natural uptake system for metalloporphyrins. As a result, we can supply different porphyrins and utilize this strain to produce proteins containing exogenous porphyrins. Co(ppIX) was synthesized as previously described, dissolved in DMSO to prepare a 10 mM stock solution, and further diluted to 2 mM with a mixture of 100 mM NaOH and 200 mM Na_2HPO_4 (pH = 12). The final Co(ppIX) stock was directly added to expression cultures at a concentration of 9 μM. Nissle 1917 competent cells were transformed with expression plasmids encoding VHb variants, and positive transformants were selected on Luria-Bertani agar plates containing ampicillin (100 μg/mL). Isolated colonies were inoculated into 5 mL centrifuge tube containing Luria-Bertani media supplemented with ampicillin and cultured at 37°C for 12 h. The starter cultures were then transferred to 1 L Luria-Bertani media (ampicillin-supplemented), and incubated at 37°C with shaking (180 rpm). After adding the Co(ppIX) solution, the cultures were induced via the anaerobic promoter, maintained under anaerobic conditions, and shaken at 110 rpm at 25°C for 30 h. Cell cultures were harvested by centrifugation at 5000 rpm. The overall pelleted bacteria were resuspended in 50 mL of 20 mM phosphate buffer (pH = 7.4). After sonication for 30 min on ice, the cell lysates were centrifuged at 12,000 rpm for 30 min to clarify the supernatant, which was subsequently used for protein purification.

Protein Purification.

The clarified lysate was transferred to a Ni-NTA column equilibrated with Ni-NTA Lysis Buffer. The resin was washed with 50 mL of Ni-NTA Lysis Buffer and then 50 mL of Ni-NTA Wash Buffer (20 mM phosphate buffer, 20 mM imidazole, pH = 7.4). Proteins were eluted with Ni-NTA Elution Buffer (20 mM phosphate buffer, 250 mM imidazole, pH = 7.4). After elution from the Ni-NTA column, the protein was loaded into a 5ml PD10 desalting column to remove imidazole. The concentration of the protein was tested by NanoDrop.

Detection of H_2O_2 in the reaction mixture

Under standard reaction conditions, O_2 used as oxidant under aerobic conditions, VHb-Co (H85Y, P54C) as catalyst (Co(ppIX) containing 0.05 mol%), **1a**, 0.05 mmol, **2a**, 0.05 mmol, PBS (50 mM, 10 % DMSO v/v), when the reaction had proceeded for 8 h, the reaction system was centrifuged. After centrifugation, the reaction system was centrifuged at 12,000 rpm for 1 min, a 100 μL solution of Mohr's salt (10 mg in 100 μL H_2O) was added to the reaction mixture, the rapid formation of ferric hydroxide flocs was immediately observed. After further centrifugation, obvious precipitates

could be observed.

Molecular Docking Analysis.

The initial structure of VHB was taken from PDB code of 3TM3, structures of VHb-Co (H85Y, P54C) was obtained by SWISS-MODEL3 using homology modeling strategy. Intermediate II was docked into the active site of WT-VHb-Co and VHb-Co (H85Y, P54C) using AutoDock Vina tool in Chimera respectively. The interactions between VHb-Co and intermediate II were showed in 2D diagram using Discovery Studio Visualizer 4.0.

Synthetic Procedures.

Preparative scale procedure for enzymatic synthesis of products 3:

To a 5 L Erlenmeyer flask containing 2.5 L of a suspension of VHb-Co (H85Y, P54C) expressing cells (*E. coli* Nissle 1917, OD_{600}=200). Following expression, the cultures were centrifuged at 4 °C and 4,000 rpm for 30 min. The cell pellets were then resuspended in phosphate buffer (10 mM, pH = 7.4, 25 mL per pellet), and the cell suspensions were combined. Then the lysed by sonication of 25 mL resuspended whole cells in 10 mM phosphate buffer (pH = 7.4) on ice for 1.5 minutes at 40% amplitude (2 second on, 3 second off) using a sonicator and a 1/8-inch tip. The suspension of VHb-Co (H85Y, P54C) was concentrated to 3 mg/ml, and then 900 μL was added to 100 μL 2-hydrazinopyridine and isothiocyanate stock solution (5 M, 0.5 mmol in DMSO). The flask was sealed with a rubber septum and sparged with O_2 for 15 min. The reaction mixture was stirred 12 h at room temperature under positive oxygen pressure. The desired product was extracted with EtOAc (3 × 5 mL) and washed with water and brine. The organic layers were combined and dried over sodium sulfate, evaporated under reduced pressure, and the residue was purified by column chromatography (hexanes/EtOAc) to afford the product. The yields of products were determined by HPLC.

Supporting Experimental Tables and Figures

Table S1. Optimization of Reaction Conditions.

Entry	Protein	Solvent	Time	pH	Yield (%)	TON
1	VHb-Co (0.05 mol%)	PBS	10 h	7.4	38	760
2	VHb-Co (0.05 mol%)	MeOH	10 h	7.4	7	140
3	VHb-Co (0.05 mol%)	MeCN	10 h	7.4	10	200
4	VHb-Co (0.05 mol%)	DMSO	10 h	7.4	14	280
5	VHb-Co (0.05 mol%)	PBS (10 % DMSO)	10 h	7.4	41	820
6	VHb-Co (0.05 mol%)	PBS (10 % DMSO)	12 h	7.4	45	900
7	VHb-Co (0.05 mol%)	PBS (10 % DMSO)	14 h	7.4	45	900
8	VHb-Co (0.05 mol%)	PBS (10 % DMSO)	16 h	7.4	45	900
9	VHb-Co (0.05 mol%)	PBS (10 % DMSO)	24 h	7.4	45	900
10	VHb-Co (0.01 mol%)	PBS (10 % DMSO)	12 h	7.4	13	260
11	VHb-Co (0.03 mol%)	PBS (10 % DMSO)	12 h	7.4	30	600
12	VHb-Co (0.07 mol%)	PBS (10 % DMSO)	12 h	7.4	45	900
13	VHb-Co (0.05 mol%)	PBS (10 % DMSO)	12 h	6.6	22	440
14	VHb-Co (0.05 mol%)	PBS (10 % DMSO)	12 h	7.0	34	680
15	VHb-Co (0.05 mol%)	PBS (10 % DMSO)	12 h	7.8	33	660

16	VHb-Co (0.05 mol%)	PBS (10 % DMSO)	12 h	8.2	19	380
17	——	PBS (10 % DMSO)	12 h	7.4	——	——
18	Co(ppIX) (0.05 mol%)	PBS (10 % DMSO)	12 h	7.4	——	——
19	VHb (0.05 mol%)	PBS (10 % DMSO)	12 h	7.4	16	320
20	VHb-Zn (0.05 mol%)	PBS (10 % DMSO)	12 h	7.4	Trace	——
21	VHb-Mn (0.05 mol%)	PBS (10 % DMSO)	12 h	7.4	29	580

Table S2. Screening of VHb mutants.

Entry	Protein	Yield (%)	TON
1	VHb-Co (H85C)	12	240
2	VHb-Co (H85Y)	61	1220
3	VHb-Co (H85S)	18	460
4	VHb-Co (H85M)	6	120
5	VHb-Co (H85A)	——	——
6	VHb-Co (H85V)	——	——
7	VHb-Co (H85Y, Q53H)	39	780
8	VHb-Co (H85Y, Q53R)	43	860
9	VHb-Co (H85Y, Q53P)	44	880
10	VHb-Co (H85Y, Q53G)	30	600
11	VHb-Co (H85Y, Q53W)	21	420
12	VHb-Co (H85Y, P54L)	66	1320
13	VHb-Co (H85Y, P54C)	84	1680
14	VHb-Co (H85Y, P54G)	57	1140
15	VHb-Co (H85Y, P54N)	62	1240
16	VHb-Co (H85Y, P54H)	50	1000
17	VHb-Co (H85Y, L57A)	24	480
18	VHb-Co (H85Y, L57Q)	28	560
19	VHb-Co (H85Y, L57R)	25	500
20	VHb-Co (H85Y, L57D)	19	380
21	VHb-Co (H85Y, L57P)	8	160
22	VHb-Co (H85Y, F43P)	57	1140
23	VHb-Co (H85Y, F43V)	46	920
24	VHb-Co (H85Y, F43A)	37	740
25	VHb-Co (H85Y, F43R)	35	700
26	VHb-Co (H85Y, F43G)	27	540
27	VHb-Co (H85Y, Y29G)	29	580
28	VHb-Co (H85Y, Y29A)	32	640
29	VHb-Co (H85Y, Y29H)	38	760
30	VHb-Co (H85Y, Y29C)	32	640
31	VHb-Co (H85Y, Y29L)	30	600
32	VHb-Co (H85Y, Y29W)	36	720

Table S3. Green chemistry metrics for enzyme system and chemical catalysis[3].*

Parameter	VHb-Co (H85Y, P54C)	TBAF
Yield (%)	84% (for 3**a**)	20%
Reaction Conditions	r.t., aqueous buffer	100 °C, organic solvent
Catalyst Loading	VHb containing 0.05 mol% Co (ppIX)	TBAF (1.1 equiv.)
Additive	none	none
PMI	1.7	384.7
E-Factor	0.3	378.9
Solvent Toxicity	Low (water/DMSO)	High (Acetonitrile)

*Water and buffer components were excluded from PMI/E-Factor calculations due to their low environmental impact and role as reaction media.

PMI (Process Mass Intensity) = Total Mass of Inputs (kg) / Mass of Product (kg)
Calculation for VHb-Co (H85Y, P54C) (0.2 mmol scale):
Substrate (**1a**): 21.8 mg
Substrate (**2a**): 27.1 mg
Enzyme (VHb-Co (H85Y, P54C)): 10.8 mg.
Total Inputs: 21.8 mg + 27.1 mg + 10.8 mg = 59.7 mg.
Product (3a): 84% yield → 35.6 mg.
PMI = 59.7/35.6 = 1.7

Comparison to Metal Catalysis (0.2 mmol scale):
Substrate (**1a**): 21.8 mg
Substrate (**2a**): 27.1 mg
TBAF Catalyst: 1.1 equiv. → 69.4 mg
Acetonitrile Solvent: 3144 mg.
Total Inputs: 21.8 + 27.1 + 69.4 + 3144 = 3262.3 mg.
Product: 20% yield → 8.48 mg.
PMI: 3262.3/8.48 = 384.7

E-Factor=Mass of Waste (kg) / Mass of Product (kg)
Waste: Enzyme = 10.8 mg.
E-Factor $_{\text{(This work)}}$: 10.8/35.6 = 0.3

Waste: TBAF + Acetonitrile = 3213.4 mg.
E-Factor $_{\text{(Previous work)}}$: 3213.4/8.48 =378.9

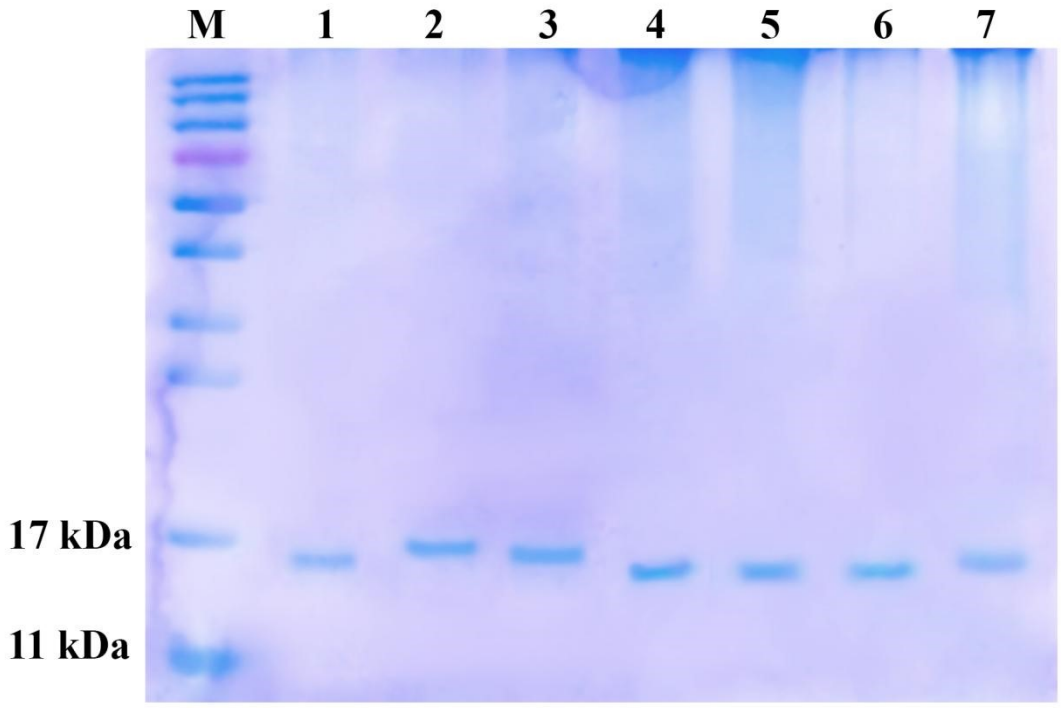


Figure S1. Characterization of purified proteins by SDS-PAGE gel electrophoresis. Protein profiles were compared against a standard protein ladder for molecular weight validation. 1: WT VHb; 2: VHb(P54C); 3: VHb(P54H); 4: VHb(P54K); 5: VHb(H85Y P54C); 6: VHb(H85S P54C); 7: VHb(H85C P54C)

Nucleotide and amino acid sequences of VHb variants

Nucleotide sequence of VHb (H85Y)

ATGTTAGACCAGCAAACCATTAACATCATCAAAGCCACTGTTCCTGTATTGAAGGAG

CATGGCGTTACCATTACCACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAG

TACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCAGCCTAAGGCTTTGGC

GATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTATTTTGCCTGC

GGTCAAAAAAATTGCAGTCAAATATTGTCAAGCAGGCGTGGCAGCAGCGCATTATCC

GATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGATGCCGCAAC

CGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATT

CAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAACATCATCATCATCATCATTAA

Amino acid sequence of VHb (H85Y)

MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFDMGRQESLEQPKALAMT

VLAAAQNIENLPAILPAVKKIAVKYCQAGVAAAHYPIVGQELLGAIKEVLGDAATDDILD

AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*

Nucleotide sequence of VHb (H85S)

ATGTTAGACCAGCAAACCATTAACATCATCAAAGCCACTGTTCCTGTATTGAAGGAG

CATGGCGTTACCATTACCACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAG

TACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCAGCCTAAGGCTTTGGC

GATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTATTTTGCCTGC

GGTCAAAAAAATTGCAGTCAAAAGCTGTCAAGCAGGCGTGGCAGCAGCGCATTATCC

GATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGATGCCGCAAC

CGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATT

CAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAACATCATCATCATCATCATTAA

Amino acid sequence of VHb (H85S)

MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFDMGRQESLEQPKALAMT

VLAAAQNIENLPAILPAVKKIAVKSCQAGVAAAHYPIVGQELLGAIKEVLGDAATDDILD

AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*

Nucleotide sequence of VHb (H85C)

ATGTTAGACCAGCAAACCATTAACATCATCAAAGCCACTGTTCCTGTATTGAAGGAG

CATGGCGTTACCATTACCACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAG

TACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCAGCCTAAGGCTTTGGC

GATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTATTTTGCCTGC

GGTCAAAAAAATTGCAGTCAAATGCTGTCAAGCAGGCGTGGCAGCAGCGCATTATCC

GATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGATGCCGCAAC

CGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATT

CAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAACATCATCATCATCATCATTAA

Amino acid sequence of VHb (H85C)

MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFDMGRQESLEQPKALAMT

VLAAAQNIENLPAILPAVKKIAVKCCQAGVAAAHYPIVGQELLGAIKEVLGDAATDDILD

AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*

Nucleotide sequence of VHb (H85M)

ATGTTAGACCAGCAAACCATTAACATCATCAAAGCCACTGTTCCTGTATTGAAGGAG

CATGGCGTTACCATTACCACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAG

TACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCAGCCTAAGGCTTTGGC

GATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTATTTTGCCTGC

GGTCAAAAAAATTGCAGTCAAAATGTGTCAAGCAGGCGTGGCAGCAGCGCATTATCC

GATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGATGCCGCAAC

CGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATT

CAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAACATCATCATCATCATCATTAA

Amino acid sequence of VHb (H85M)

MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFDMGRQESLEQPKALAMT

VLAAAQNIENLPAILPAVKKIAVKMCQAGVAAAHYPIVGQELLGAIKEVLGDAATDDILD

AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*

Nucleotide sequence of VHb (H85A)

ATGTTAGACCAGCAAACCATTAACATCATCAAAGCCACTGTTCCTGTATTGAAGGAG

CATGGCGTTACCATTACCACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAG

TACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCAGCCTAAGGCTTTGGC

GATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTATTTTGCCTGC

GGTCAAAAAAATTGCAGTCAAAGCTTGTCAAGCAGGCGTGGCAGCAGCGCATTATCC

GATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGATGCCGCAAC

CGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATT

CAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAACATCATCATCATCATCATTAA

Amino acid sequence of VHb (H85A)

MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFDMGRQESLEQPKALAMT

VLAAAQNIENLPAILPAVKKIAVKACQAGVAAAHYPIVGQELLGAIKEVLGDAATDDILD

AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*

Nucleotide sequence of VHb (H85Y, Q53H)

ATGTTAGACCAGCAAACCATTAACATCATCAAAGCCACTGTTCCTGTATTGAAGGAG

CATGGCGTTACCATTACCACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAG

TACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCATCCTAAGGCTTTGGC

GATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTATTTTGCCTGC

GGTCAAAAAAATTGCAGTCAAATATTGTCAAGCAGGCGTGGCAGCAGCGCATTATCC

GATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGATGCCGCAAC

CGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATT

CAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAACATCATCATCATCATCATTAA

Amino acid sequence of VHb (H85Y, Q53H)

MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFDMGRQESLEHPKALAMT

VLAAAQNIENLPAILPAVKKIAVKYCQAGVAAAHYPIVGQELLGAIKEVLGDAATDDILD

AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*

Nucleotide sequence of VHb (H85Y, Q53R)

ATGTTAGACCAGCAAACCATTAACATCATCAAAGCCACTGTTCCTGTATTGAAGGAG

CATGGCGTTACCATTACCACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAG

TACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCGACCTAAGGCTTTGGC

GATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTATTTTGCCTGC

GGTCAAAAAAATTGCAGTCAAATATTGTCAAGCAGGCGTGGCAGCAGCGCATTATCC

GATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGATGCCGCAAC

CGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATT

CAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAACATCATCATCATCATCATTAA

Amino acid sequence of VHb (H85Y, Q53R)

MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFDMGRQESLERPKALAMT

VLAAAQNIENLPAILPAVKKIAVKYCQAGVAAAHYPIVGQELLGAIKEVLGDAATDDILD

AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*

Nucleotide sequence of VHb (H85Y, P54L)

ATGTTAGACCAGCAAACCATTAACATCATCAAAGCCACTGTTCCTGTATTGAAGGAG

CATGGCGTTACCATTACCACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAG

TACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCAGTTAAAGGCTTTGGC

GATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTATTTTGCCTGC

GGTCAAAAAAATTGCAGTCAAATATTGTCAAGCAGGCGTGGCAGCAGCGCATTATCC

GATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGATGCCGCAAC

CGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATT

CAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAACATCATCATCATCATCATTAA

Amino acid sequence of VHb (H85Y, P54L)

MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFDMGRQESLEQLKALAMT

VLAAAQNIENLPAILPAVKKIAVKYCQAGVAAAHYPIVGQELLGAIKEVLGDAATDDILD

AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*

Nucleotide sequence of VHb (H85Y, P54C)

ATGTTAGACCAGCAAACCATTAACATCATCAAAGCCACTGTTCCTGTATTGAAGGAG

CATGGCGTTACCATTACCACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAG

TACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCAGTGTAAGGCTTTGGC

GATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTATTTTGCCTGC

GGTCAAAAAAATTGCAGTCAAATATTGTCAAGCAGGCGTGGCAGCAGCGCATTATCC

GATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGATGCCGCAAC

CGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATT

CAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAACATCATCATCATCATCATTAA

Amino acid sequence of VHb (H85Y, P54C)

MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFDMGRQESLEQCKALAMT

VLAAAQNIENLPAILPAVKKIAVKYCQAGVAAAHYPIVGQELLGAIKEVLGDAATDDILD

AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*

Nucleotide sequence of VHb (H85Y, L57A)

ATGTTAGACCAGCAAACCATTAACATCATCAAAGCCACTGTTCCTGTATTGAAGGAG

CATGGCGTTACCATTACCACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAG

TACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCAGCCTAAGGCTGCTGC

GATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTATTTTGCCTGC

GGTCAAAAAAATTGCAGTCAAATATTGTCAAGCAGGCGTGGCAGCAGCGCATTATCC

GATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGATGCCGCAAC

CGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATT

CAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAACATCATCATCATCATCATTAA

Amino acid sequence of VHb (H85Y, L57A)

MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFDMGRQESLEQPKAAAMT

VLAAAQNIENLPAILPAVKKIAVKYCQAGVAAAHYPIVGQELLGAIKEVLGDAATDDILD

AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*

Nucleotide sequence of VHb (H85Y, L57Q)

ATGTTAGACCAGCAAACCATTAACATCATCAAAGCCACTGTTCCTGTATTGAAGGAG

CATGGCGTTACCATTACCACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAG

TACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCAGCCTAAGGCTCAGGC

GATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTATTTTGCCTGC

GGTCAAAAAAATTGCAGTCAAATATTGTCAAGCAGGCGTGGCAGCAGCGCATTATCC

GATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGATGCCGCAAC

CGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATT

CAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAACATCATCATCATCATCATTAA

Amino acid sequence of VHb (H85Y, L57Q)

MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFDMGRQESLEQPKAQAMT

VLAAAQNIENLPAILPAVKKIAVKYCQAGVAAAHYPIVGQELLGAIKEVLGDAATDDILD

AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*

Nucleotide sequence of VHb (H85Y, K55L)

ATGTTAGACCAGCAAACCATTAACATCATCAAAGCCACTGTTCCTGTATTGAAGGAG

CATGGCGTTACCATTACCACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAG

TACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCAGCCTCTGGCTTTGGC

GATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTATTTTGCCTGC

GGTCAAAAAAATTGCAGTCAAATATTGTCAAGCAGGCGTGGCAGCAGCGCATTATCC

GATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGATGCCGCAAC

CGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATT

CAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAACATCATCATCATCATCATTAA

Amino acid sequence of VHb (H85Y, K55L)

MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFDMGRQESLEQPLALAMT

VLAAAQNIENLPAILPAVKKIAVKYCQAGVAAAHYPIVGQELLGAIKEVLGDAATDDILD

AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*

Nucleotide sequence of VHb (H85Y, K55D)

ATGTTAGACCAGCAAACCATTAACATCATCAAAGCCACTGTTCCTGTATTGAAGGAG

CATGGCGTTACCATTACCACGACTTTTTATAAAAACTTGTTTGCCAAACACCCTGAAG

TACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCAGCCTGATGCTTTGGC

GATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTATTTTGCCTGC

GGTCAAAAAAATTGCAGTCAAATATTGTCAAGCAGGCGTGGCAGCAGCGCATTATCC

GATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGATGCCGCAAC

CGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATT

CAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAACATCATCATCATCATCATTAA

Amino acid sequence of VHb (H85Y, K55D)

MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFDMGRQESLEQPDALAMT

VLAAAQNIENLPAILPAVKKIAVKYCQAGVAAAHYPIVGQELLGAIKEVLGDAATDDILD

AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*

Nucleotide sequence of VHb (H85Y, Y29G)

ATGTTAGACCAGCAAACCATTAACATCATCAAAGCCACTGTTCCTGTATTGAAGGAG

CATGGCGTTACCATTACCACGACTTTTGGTAAAAACTTGTTTGCCAAACACCCTGAAG

TACGTCCTTTGTTTGATATGGGTCGCCAAGAATCTTTGGAGCAGCCTAAGGCTTTGGC

GATGACGGTATTGGCGGCAGCGCAAAACATTGAAAATTTGCCAGCTATTTTGCCTGC

GGTCAAAAAAATTGCAGTCAAATATTGTCAAGCAGGCGTGGCAGCAGCGCATTATCC

GATTGTCGGTCAAGAATTGTTGGGTGCGATTAAAGAAGTATTGGGCGATGCCGCAAC

CGATGACATTTTGGACGCGTGGGGCAAGGCTTATGGCGTGATTGCAGATGTGTTTATT

CAAGTGGAAGCAGATTTGTACGCTCAAGCGGTTGAACATCATCATCATCATCATTAA

Amino acid sequence of VHb (H85Y, Y29G)

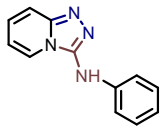
MLDQQTINIIKATVPVLKEHGVTITTTFGKNLFAKHPEVRPLFDMGRQESLEQPKALAMT

VLAAAQNIENLPAILPAVKKIAVKYCQAGVAAAHYPIVGQELLGAIKEVLGDAATDDILD

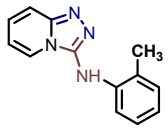
AWGKAYGVIADVFIQVEADLYAQAVEHHHHHH*

NMR Data of Products

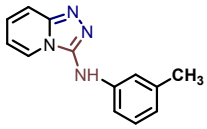
All the compounds obtained were known compounds[4].



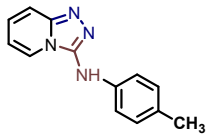
N-phenyl-[1,2,4]triazolo[4,3-a]pyridin-3-amine (**3a**), Yield:84%, 88.2mg, light-yellow solid, purified by flash column chromatography (EA: MeOH = 10:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.23 (s, 1H), 8.34 (d, J = 7.1 Hz, 1H), 7.60 (d, J = 9.3 Hz, 1H), 7.55 (d, J = 7.5 Hz, 2H), 7.32 – 7.28 (m, 2H), 7.23 (dd, J = 8.9, 5.8 Hz, 1H), 6.90 (q, J = 6.9 Hz, 2H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{12}H_{11}N_4$: 211.0978; Found: 211.0974.



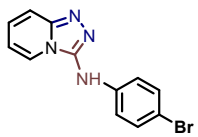
N-(o-tolyl)-[1,2,4]triazolo[4,3-a]pyridin-3-amine (**3b**), Yield:63%, 70.6mg, light-yellow solid, purified by flash column chromatography (EA: MeOH = 10:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.16 (s, 1H), 8.10 (d, J = 7.0 Hz, 1H), 7.64 (d, J = 9.3 Hz, 1H), 7.27 (dd, J = 9.4, 6.5 Hz, 1H), 7.18 (d, J = 7.6 Hz, 1H), 7.09 – 7.04 (m, 1H), 6.99 (d, J = 7.2 Hz, 1H), 6.90 – 6.85 (m, 2H), 2.28 (s, 3H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{13}H_{13}N_4$: 225.1135; Found: 225.1132.



N-(m-tolyl)-[1,2,4]triazolo[4,3-a]pyridin-3-amine (**3c**), Yield:66%, 73.9mg, white solid, purified by flash column chromatography (EA: MeOH = 10:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.14 (s, 1H), 8.33 (d, J = 7.1 Hz, 1H), 7.62 (d, J = 9.3 Hz, 1H), 7.40 (s, 1H), 7.32 (d, J = 8.8 Hz, 1H), 7.25 – 7.16 (m, 2H), 6.90 (t, J = 6.2 Hz, 1H), 6.74 (d, J = 7.4 Hz, 1H), 2.30 (s, 3H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{13}H_{13}N_4$: 225.1135; Found: 225.1130.

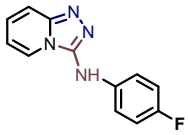


N-(p-tolyl)-[1,2,4]triazolo[4,3-a]pyridin-3-amine (**3d**), Yield:72%, 80.6mg, off-white solid, purified by flash column chromatography (EA: MeOH = 10:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.13 (s, 1H), 8.33 (d, J = 7.1 Hz, 1H), 7.60 (d, J = 9.3 Hz, 1H), 7.47 (d, J = 8.4 Hz, 2H), 7.25 – 7.20 (m, 1H), 7.12 (d, J = 8.3 Hz, 2H), 6.88 (t, J = 6.7 Hz, 1H), 2.26 (s, 3H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{13}H_{13}N_4$: 225.1135; Found: 225.1131.

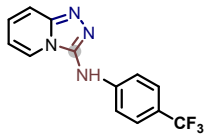


N-(4-bromophenyl)-[1,2,4]triazolo[4,3-a]pyridin-3-amine (**3e**), Yield:73%, 105.4mg, white solid,

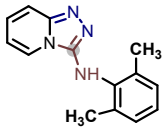
purified by flash column chromatography (EA: MeOH = 30:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.43 (s, 1H), 8.34 (d, J = 7.1 Hz, 1H), 7.63 (d, J = 9.4 Hz, 1H), 7.56 (s, 2H), 7.48 (d, J = 8.9 Hz, 2H), 7.27 – 7.24 (m, 1H), 6.92 (t, J = 6.2 Hz, 1H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{12}H_{10}BrN_4$: 289.0083; Found: 289.0079.



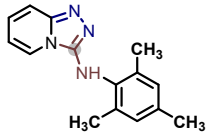
N-(4-fluorophenyl)-[1,2,4]triazolo[4,3-a]pyridin-3-amine (**3f**), Yield:70%, 79.8mg, white solid, purified by flash column chromatography (EA: MeOH = 12:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.29 (s, 1H), 8.34 (d, J = 7.0 Hz, 1H), 7.65 – 7.59 (m, 3H), 7.24 (dd, J = 8.8, 5.9 Hz, 1H), 7.17 (t, J = 8.9 Hz, 2H), 6.90 (t, J = 6.2 Hz, 1H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{12}H_{10}FN_4$: 229.0884; Found: 229.0878.



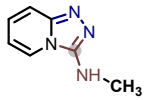
N-(4-(trifluoromethyl)phenyl)-[1,2,4]triazolo[4,3-a]pyridin-3-amine (**3g**), Yield:68%, 94.5mg, yellow solid, purified by flash column chromatography (EA: MeOH = 12:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.75 (s, 1H), 8.38 (d, J = 7.0 Hz, 1H), 7.73 (d, J = 8.7 Hz, 2H), 7.68 (d, J = 9.0 Hz, 3H), 7.30 (dd, J = 9.5, 6.3 Hz, 1H), 6.96 (t, J = 6.7 Hz, 1H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{13}H_{10}F_3N_4$: 279.0852; Found: 279.0848.



N-(2,6-dimethylphenyl)-[1,2,4]triazolo[4,3-a]pyridin-3-amine (**3h**), Yield:35%, 41.6mg, light-yellow solid, purified by flash column chromatography (EA: MeOH = 12:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.27 (d, J = 7.0 Hz, 1H), 8.13 (s, 1H), 7.53 (d, J = 9.4 Hz, 1H), 7.19 (dd, J = 9.2, 6.5 Hz, 1H), 7.11 (d, J = 6.2 Hz, 2H), 7.08 – 7.04 (m, 1H), 6.87 (t, J = 6.7 Hz, 1H), 2.10 (s, 6H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{14}H_{15}N_4$: 239.1291; Found: 239.1286.

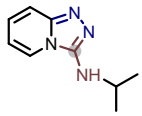


N-mesityl-[1,2,4]triazolo[4,3-a]pyridin-3-amine(**3i**), Yield:24%, 30.2mg, light-yellow solid, purified by flash column chromatography (EA: MeOH = 10:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.24 (d, J = 7.1 Hz, 1H), 8.05 (s, 1H), 7.62 (d, J = 6.7 Hz, 1H), 6.92 (s, 2H), 6.84 (t, J = 6.5 Hz, 1H), 6.66 (d, J = 8.2 Hz, 1H), 2.25 (s, 3H), 2.07 (s, 6H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{15}H_{17}N_4$: 253.1448; Found: 253.1445.

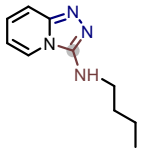


N-methyl-[1,2,4]triazolo[4,3-a]pyridin-3-amine(**3j**), Yield:62%, 45.8mg, white solid, purified by

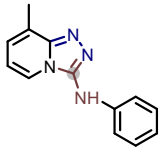
flash column chromatography (EA: MeOH = 5:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.03 (d, J = 7.1 Hz, 1H), 7.45 (d, J = 9.4 Hz, 1H), 7.12 – 7.07 (m, 1H), 6.75 (t, J = 6.7 Hz, 1H), 6.63 (d, J = 5.2 Hz, 1H), 2.99 (d, J = 4.9 Hz, 3H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_7H_9N_4$: 149.0822; Found: 149.0820.



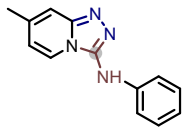
N-isopropyl-[1,2,4]triazolo[4,3-a]pyridin-3-amine(**3k**), Yield:55%, 48.4mg, brown solid, purified by flash column chromatography (EA: MeOH = 8:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.10 (d, J = 7.1 Hz, 1H), 7.44 (d, J = 9.4 Hz, 1H), 7.08 (dd, J = 10.0, 5.9 Hz, 1H), 6.75 – 6.71 (m, 1H), 6.42 (d, J = 6.9 Hz, 1H), 3.96 (h, J = 6.5 Hz, 1H), 1.29 (d, J = 6.4 Hz, 6H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_9H_{13}N_4$: 177.1135; Found: 177.1131.



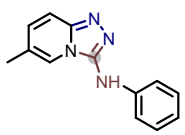
N-butyl-[1,2,4]triazolo[4,3-a]pyridin-3-amine(**3l**), Yield:51%, 48.4mg, white solid, purified by flash column chromatography (EA: MeOH = 12:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.09 (d, J = 7.0 Hz, 1H), 7.44 (d, J = 9.4 Hz, 1H), 7.08 (dd, J = 10.5, 6.3 Hz, 1H), 6.75 – 6.71 (m, 1H), 6.60 (t, J = 5.6 Hz, 1H), 3.40 (d, J = 7.2 Hz, 1H), 3.36 (s, 1H), 1.67 – 1.63 (m, 2H), 1.44 – 1.38 (m, 2H), 0.94 (t, J = 7.4 Hz, 3H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{10}H_{15}N_4$: 191.1291; Found: 191.1289.



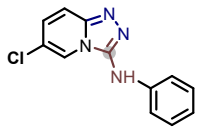
8-methyl-N-phenyl-[1,2,4]triazolo[4,3-a]pyridin-3-amine(**3m**), Yield:79%, 88.5mg, white solid, purified by flash column chromatography (EA: MeOH = 10:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.19 (s, 1H), 8.20 (d, J = 7.0 Hz, 1H), 7.54 (d, J = 7.4 Hz, 2H), 7.33 – 7.29 (m, 2H), 7.04 (d, J = 6.7 Hz, 1H), 6.91 (t, J = 7.3 Hz, 1H), 6.82 (t, J = 6.8 Hz, 1H), 2.48 (s, 3H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{13}H_{13}N_4$: 225.1135; Found: 225.1132.



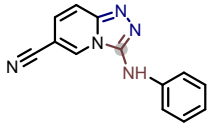
7-methyl-N-phenyl-[1,2,4]triazolo[4,3-a]pyridin-3-amine(**3n**), Yield:71%, 79.5mg, white solid, purified by flash column chromatography (EA: MeOH = 10:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.18 (s, 1H), 8.24 (d, J = 7.0 Hz, 1H), 7.54 (d, J = 7.5 Hz, 2H), 7.38 (s, 1H), 7.33 – 7.29 (m, 2H), 6.91 (t, J = 7.3 Hz, 1H), 6.77 (d, J = 5.6 Hz, 1H), 2.34 (s, 3H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{13}H_{13}N_4$: 225.1135; Found: 225.1131.



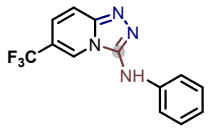
6-methyl-N-phenyl-[1,2,4]triazolo[4,3-a]pyridin-3-amine(**3o**), Yield:74%, 82.8mg, yellow solid, purified by flash column chromatography (EA: MeOH = 10:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.14 (s, 1H), 8.17 (s, 1H), 7.56 (dd, J = 13.9, 8.4 Hz, 3H), 7.34 – 7.30 (m, 2H), 7.15 (d, J = 9.4 Hz, 1H), 6.92 (t, J = 7.3 Hz, 1H), 2.29 (s, 3H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{13}H_{13}N_4$: 225.1135; Found: 225.1130.



6-chloro-N-phenyl-[1,2,4]triazolo[4,3-a]pyridin-3-amine(**3p**), Yield:73%, 89.1mg, white solid, purified by flash column chromatography (EA: MeOH = 15:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.29 (s, 1H), 8.72 (s, 1H), 7.67 (d, J = 9.8 Hz, 3H), 7.38 – 7.34 (m, 2H), 7.28 (dd, J = 9.8, 1.8 Hz, 1H), 6.97 (t, J = 7.4 Hz, 1H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{12}H_{10}ClN_4$: 245.0589; Found: 245.0586.



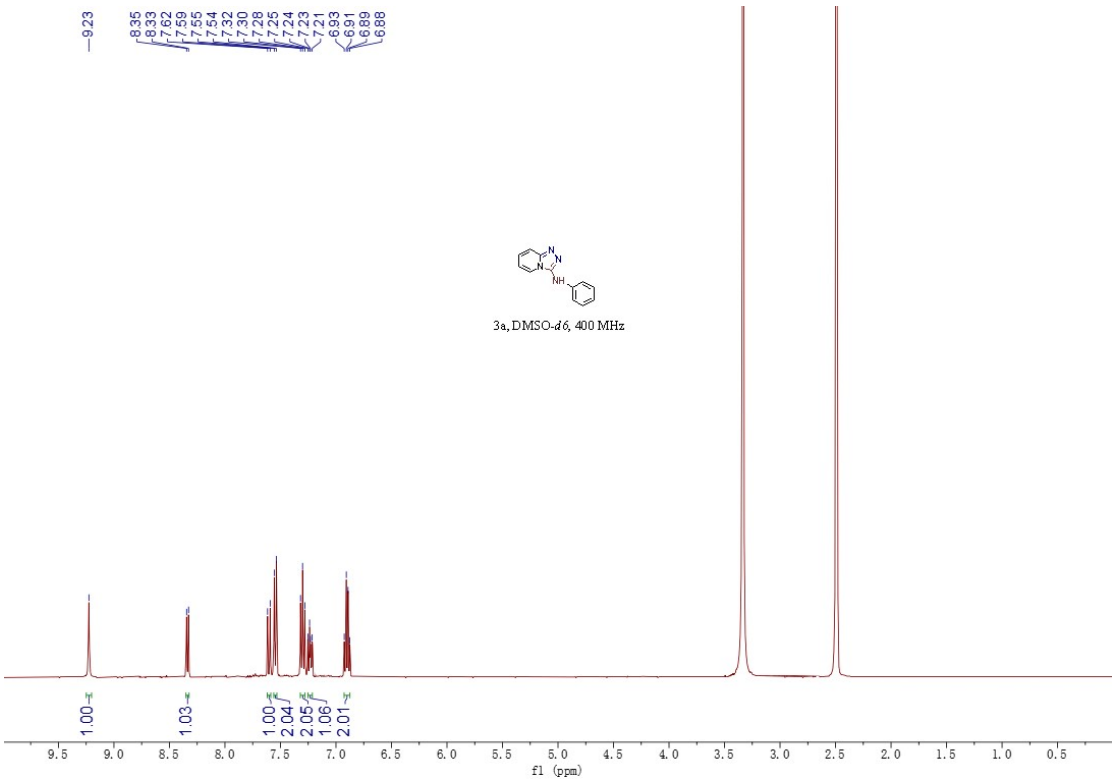
3-(phenylamino)-[1,2,4]triazolo[4,3-a]pyridine-6-carbonitrile(**3q**), Yield:34%, 39.9mg, yellow solid, purified by flash column chromatography (EA: MeOH = 12:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.45 (s, 1H), 9.25 (s, 1H), 7.76 (d, J = 9.7 Hz, 1H), 7.72 (d, J = 7.4 Hz, 2H), 7.44 – 7.36 (m, 3H), 7.00 (t, J = 7.3 Hz, 1H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{13}H_{10}N_5$: 236.0931; Found: 236.0927.



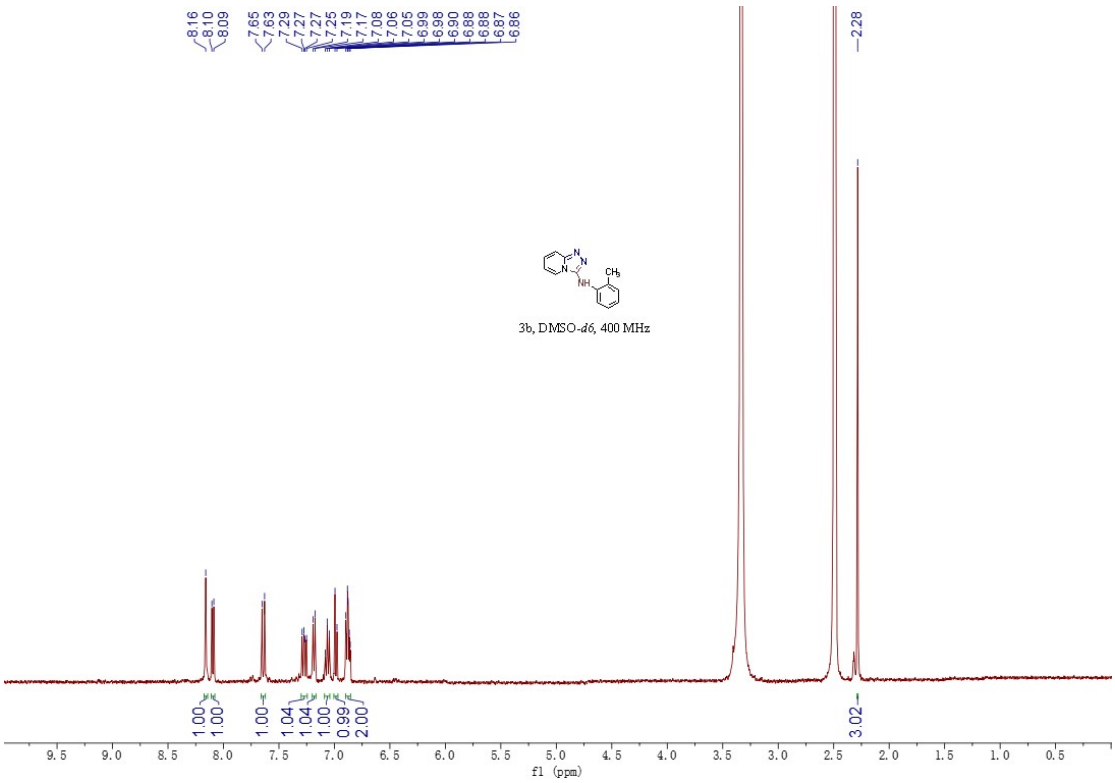
N-phenyl-6-(trifluoromethyl)-[1,2,4]triazolo[4,3-a]pyridin-3-amine(**3r**), Yield:45%, 62.5mg, white solid, purified by flash column chromatography (EA: MeOH = 12:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.53 (s, 1H), 9.14 (s, 1H), 7.80 (d, J = 9.7 Hz, 1H), 7.76 (d, J = 7.6 Hz, 2H), 7.42 (d, J = 9.7 Hz, 1H), 7.40 – 7.36 (m, 2H), 6.99 (t, J = 7.3 Hz, 1H).
HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{13}H_{10}F_3N_4$: 279.0852; Found: 279.0850.

^{1}H NMR Spectra

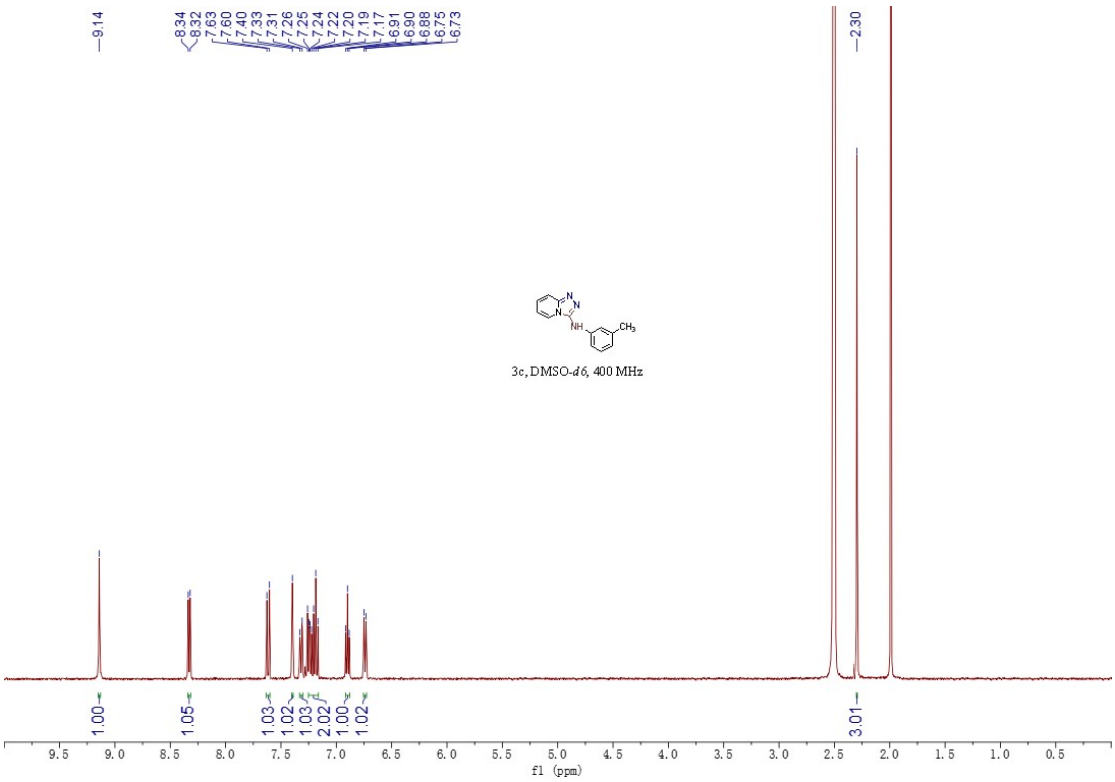
All the compounds obtained were known compounds[3,4].



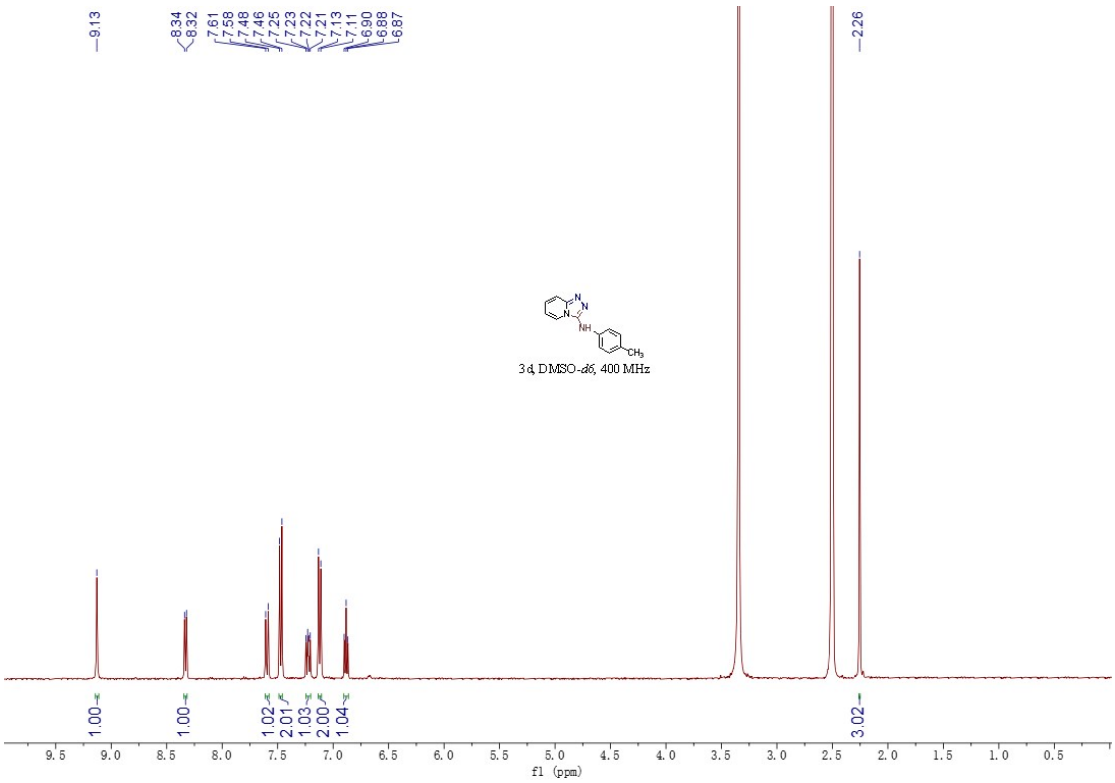
3a, DMSO-*d6*, 400 MHz



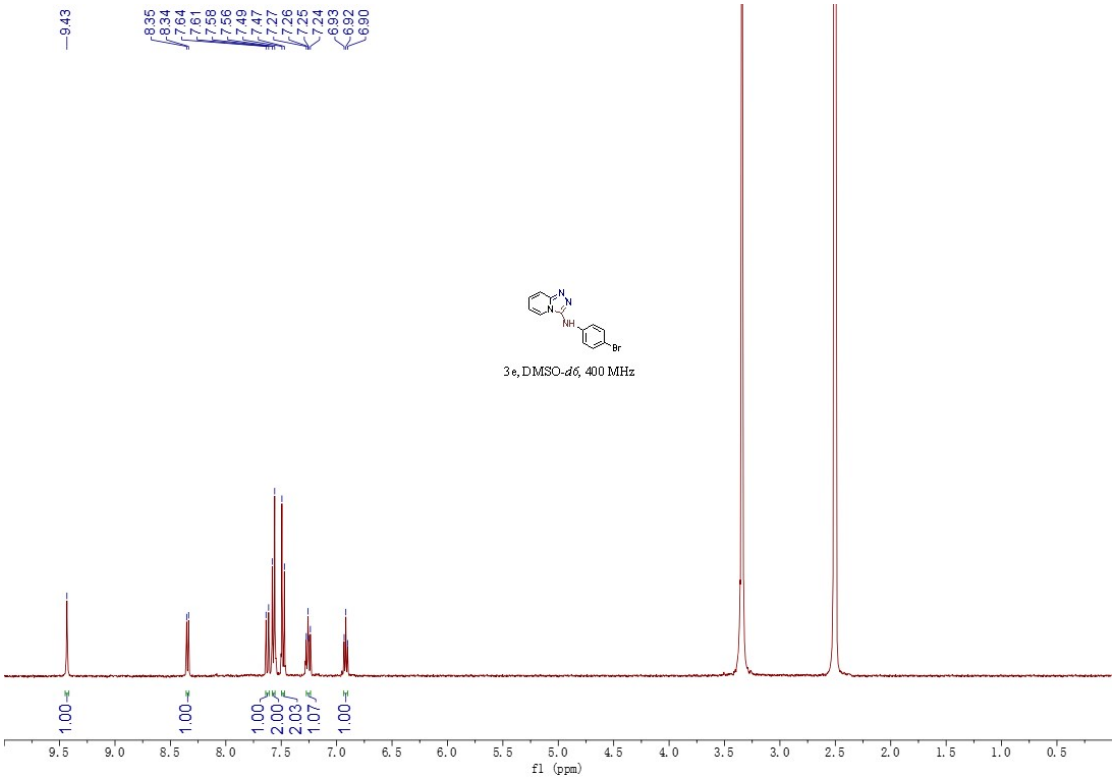
3b, DMSO-*d6*, 400 MHz

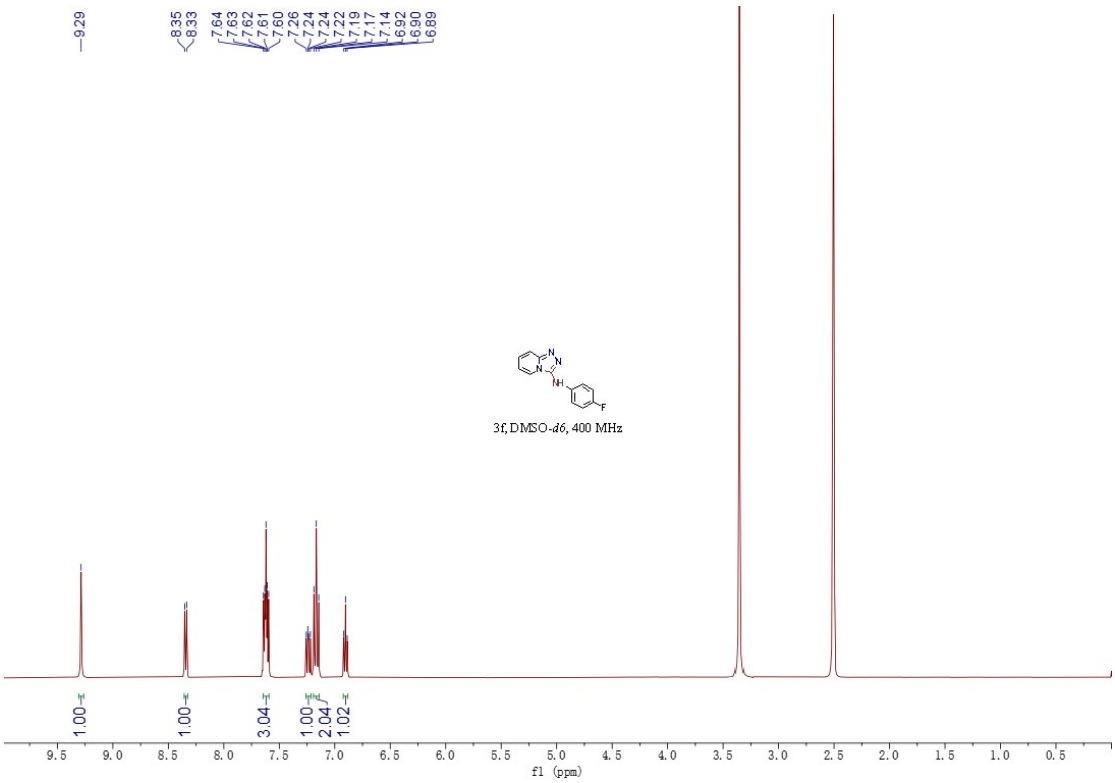


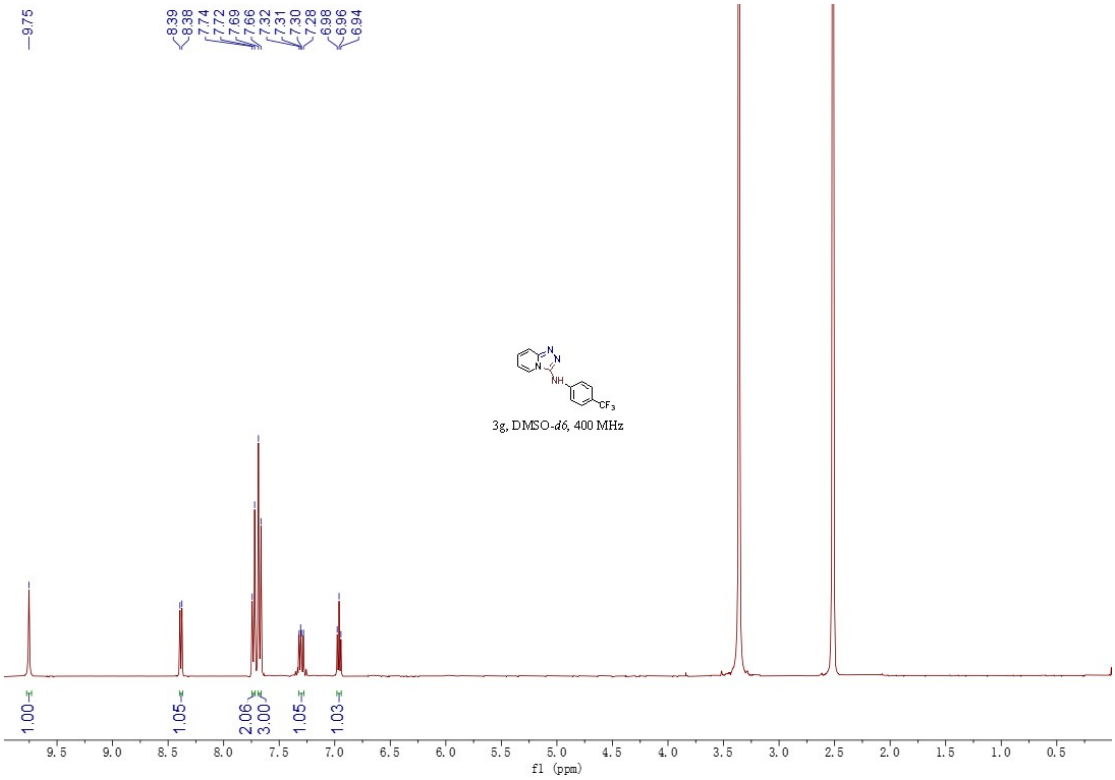
3c, DMSO-*d6*, 400 MHz

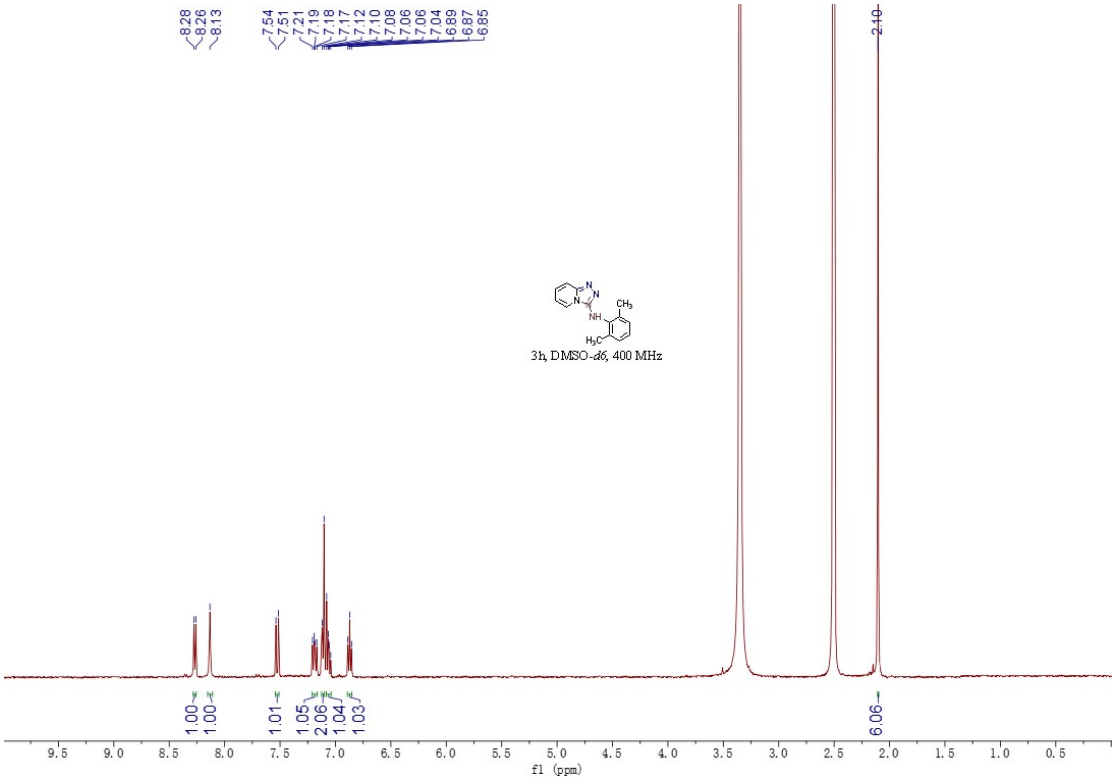


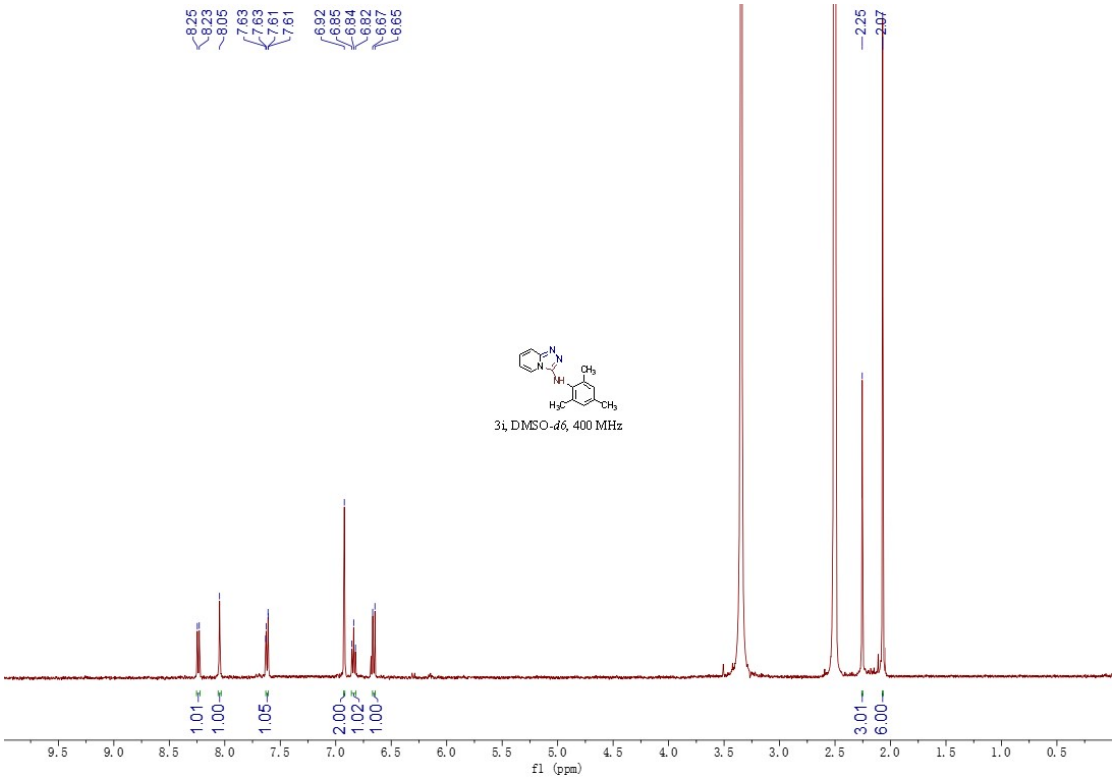
3d, DMSO-*d6*, 400 MHz

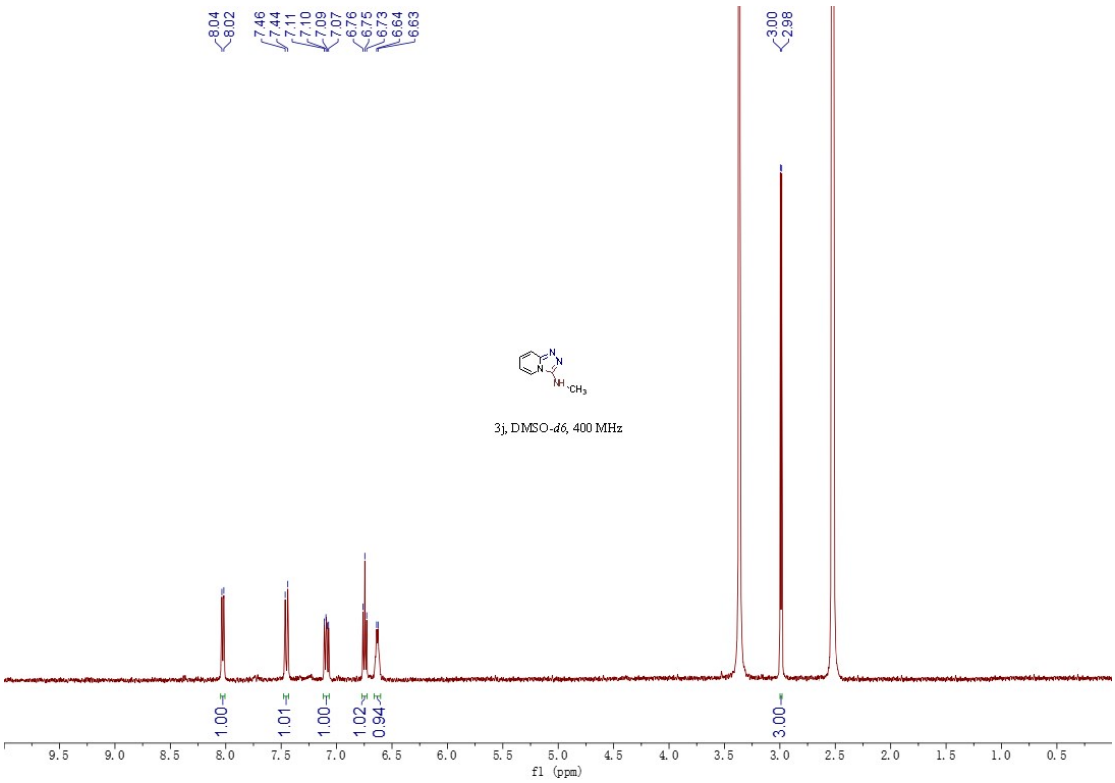

9.43
8.35
8.34
7.64
7.61
7.58
7.56
7.49
7.47
7.27
7.26
7.25
7.24
6.93
6.92
6.90
3e, DMSO-d6, 400 MHz
1.00
1.00
1.00
2.00
2.03
1.07
1.00
f1 (ppm)

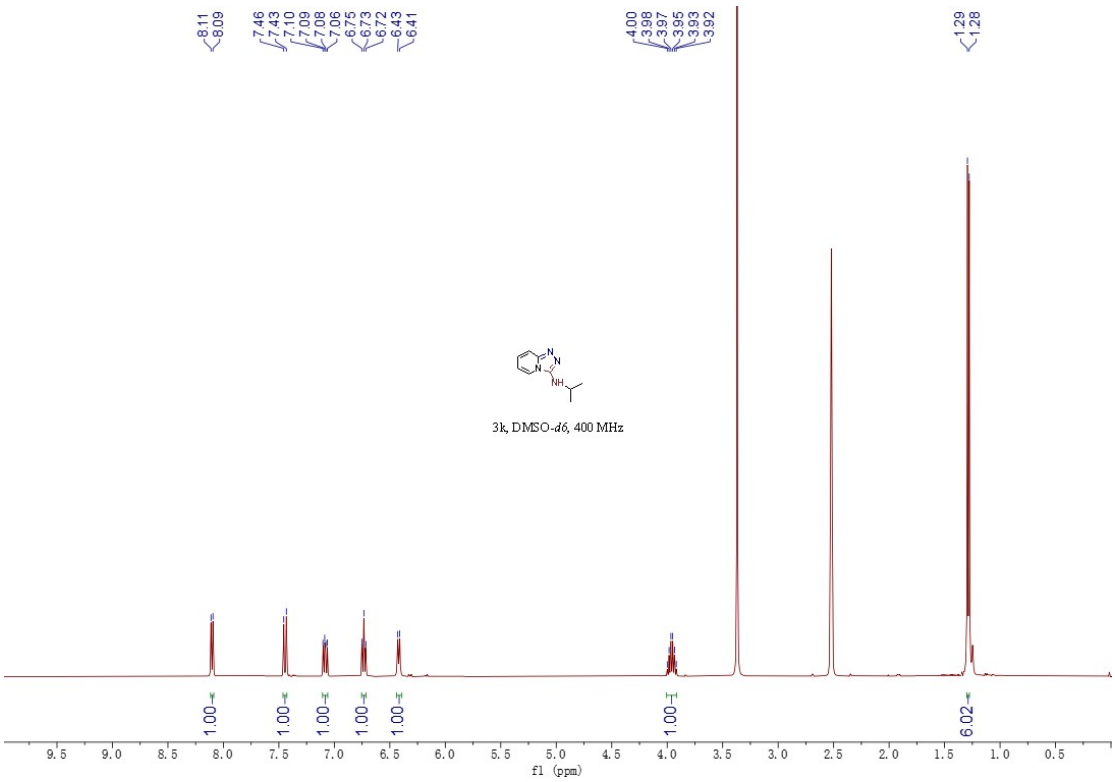

9.29
8.35
8.33
7.64
7.63
7.62
7.61
7.60
7.26
7.24
7.24
7.22
7.19
7.17
7.14
6.92
6.90
6.89
3f, DMSO-d6, 400 MHz
1.00
1.00
3.04
1.00
2.04
1.02
f1 (ppm)

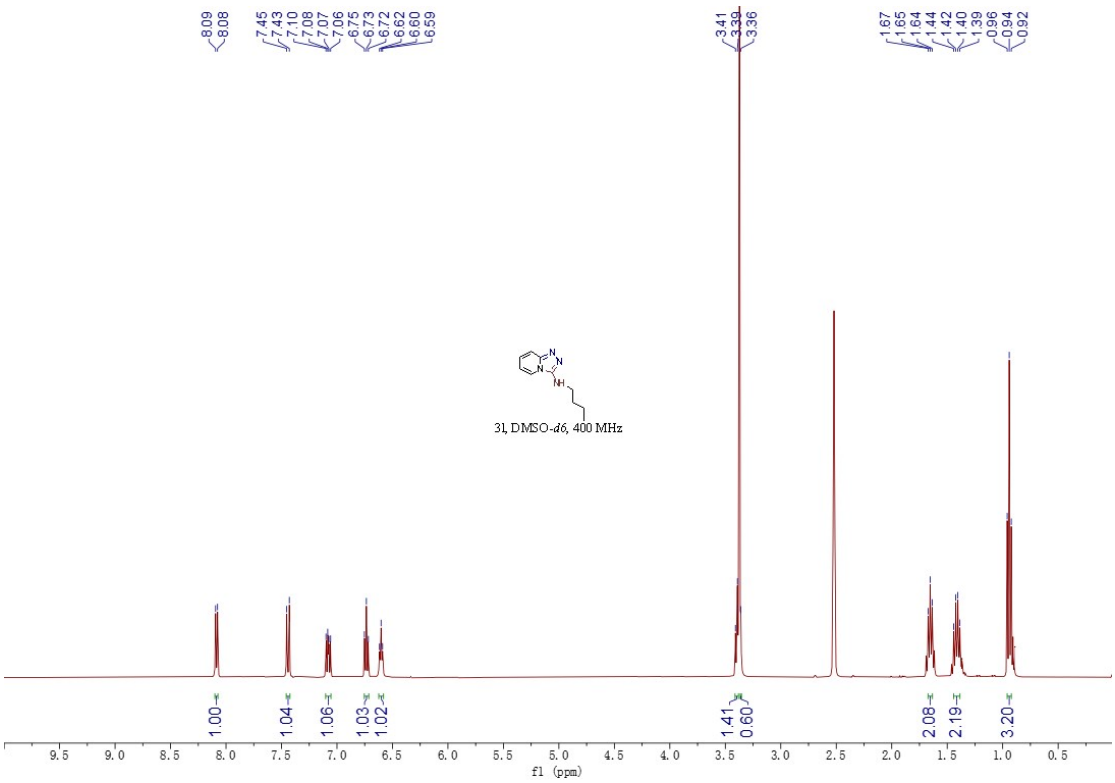

9.75
8.39
8.38
7.74
7.72
7.69
7.66
7.32
7.31
7.30
7.28
6.98
6.96
6.94
3g, DMSO-d6, 400 MHz
1.00
1.05
2.06
3.00
1.05
1.03
f1 (ppm)

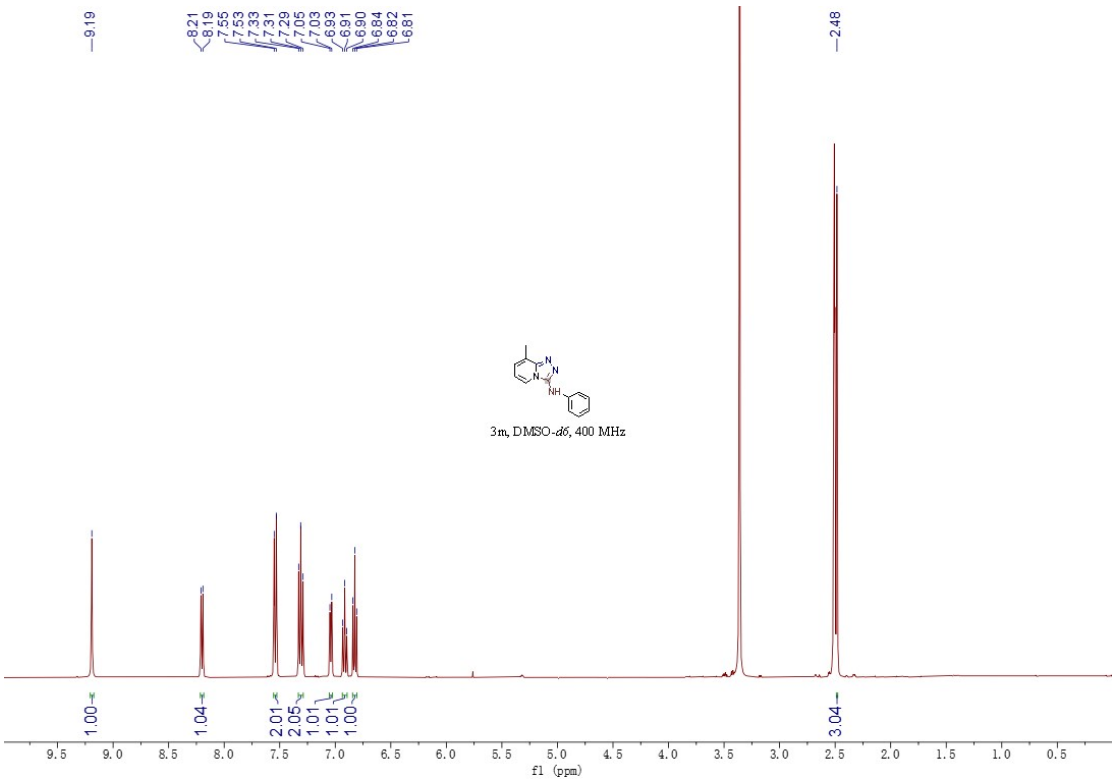

8.28
8.26
8.13
7.54
7.51
7.21
7.19
7.18
7.17
7.12
7.10
7.08
7.06
7.06
7.04
6.89
6.87
6.85
2.10
3h, DMSO-d6, 400 MHz
1.00
1.00
1.01
1.05
2.06
1.04
1.03
6.06
f1 (ppm)


8.25
8.23
8.05
7.63
7.63
7.61
7.61
6.92
6.85
6.84
6.82
6.67
6.65
2.25
2.07
3i, DMSO-d6, 400 MHz
1.01
1.00
1.05
2.00
1.02
1.00
3.01
6.00
f1 (ppm)

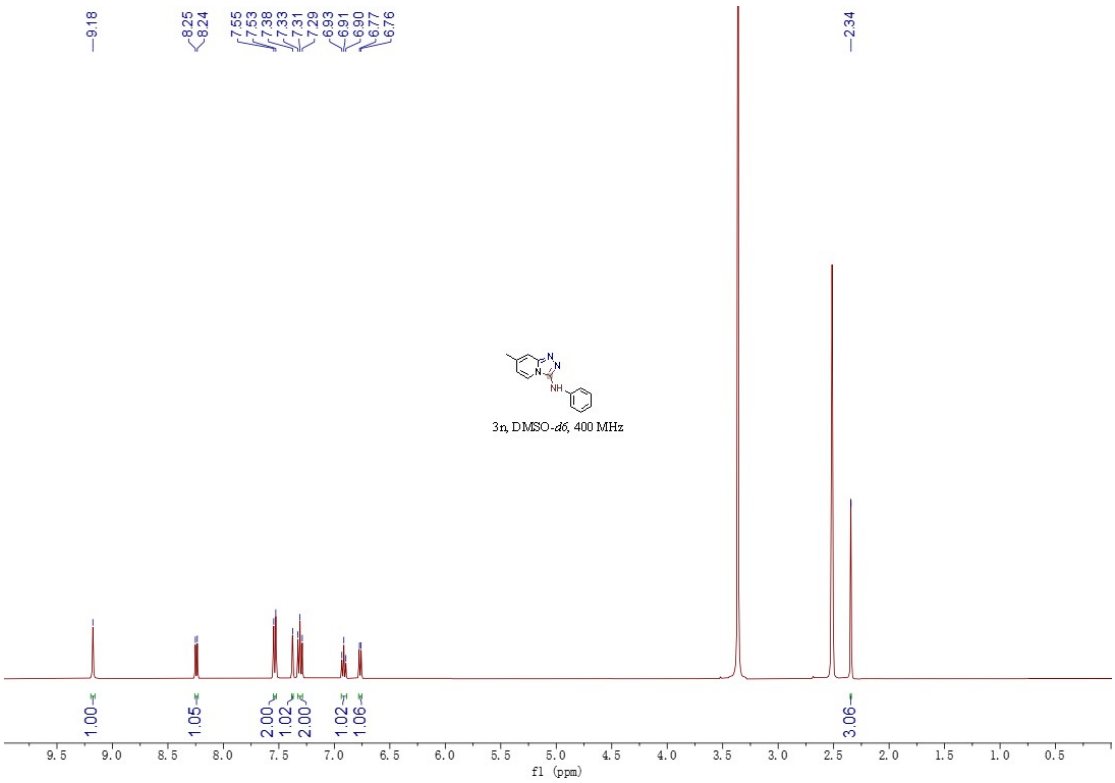

8.04
8.02
7.46
7.44
7.11
7.10
7.09
7.07
6.76
6.75
6.73
6.64
6.63
3.00
2.98
3j, DMSO-d6, 400 MHz
1.00
1.01
1.00
1.02
0.94
3.00
f1 (ppm)


8.11
8.09
7.46
7.43
7.10
7.09
7.08
7.06
6.75
6.73
6.72
6.43
6.41
4.00
3.98
3.97
3.95
3.93
3.92
1.29
1.28
3k, DMSO-d6, 400 MHz
1.00
1.00
1.00
1.00
1.00
1.00
6.02
f1 (ppm)

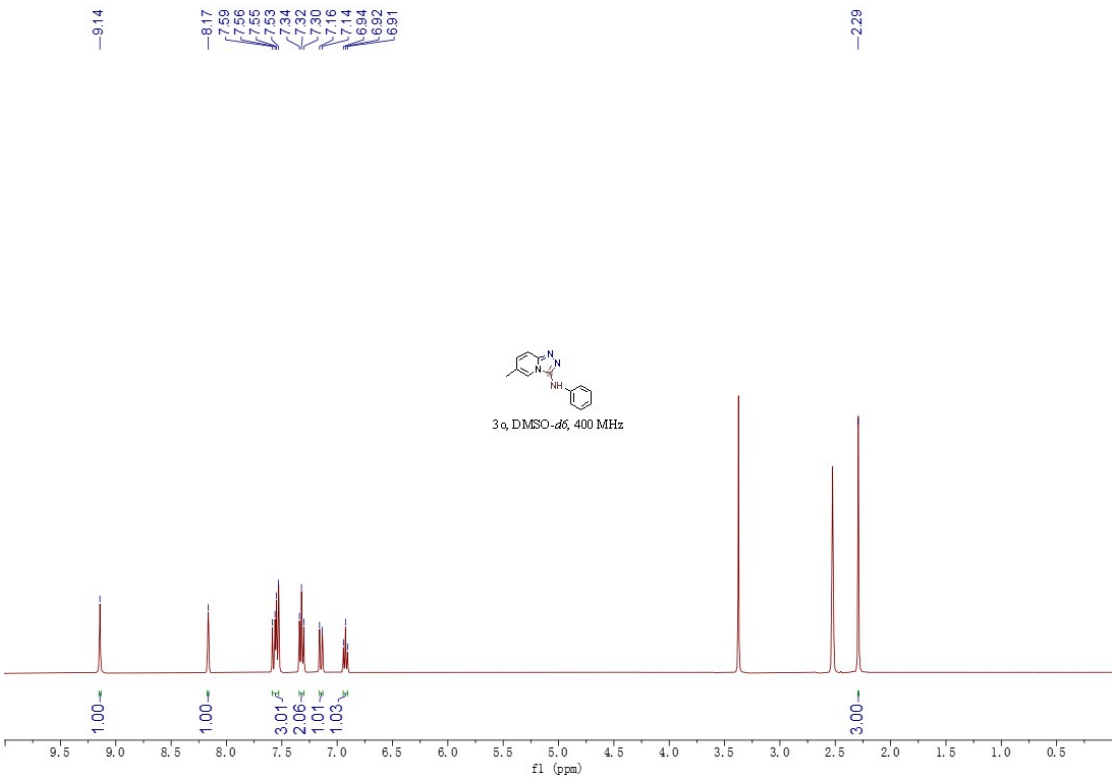

8.09
8.08
7.45
7.43
7.10
7.08
7.07
7.06
6.75
6.73
6.72
6.62
6.60
6.59
3.41
3.39
3.36
1.67
1.65
1.64
1.44
1.42
1.40
1.39
0.96
0.94
0.92
3l, DMSO-d6, 400 MHz
1.00
1.04
1.06
1.03
1.02
1.41
0.60
2.08
2.19
3.20
f1 (ppm)

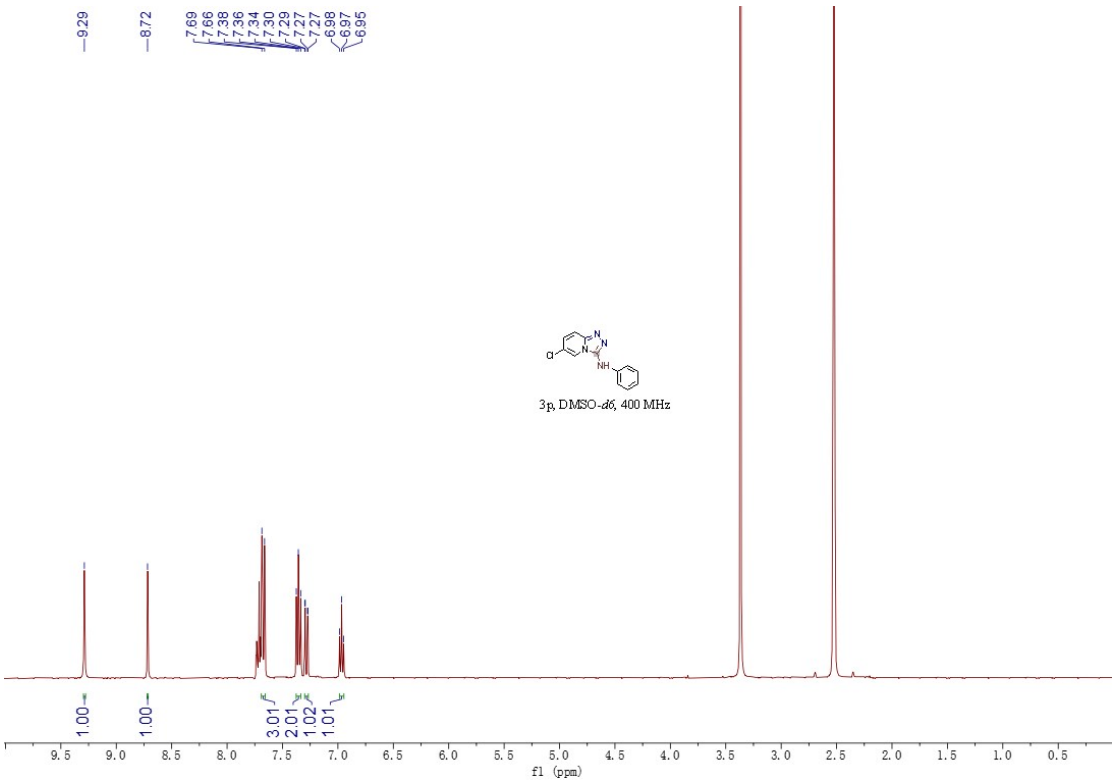


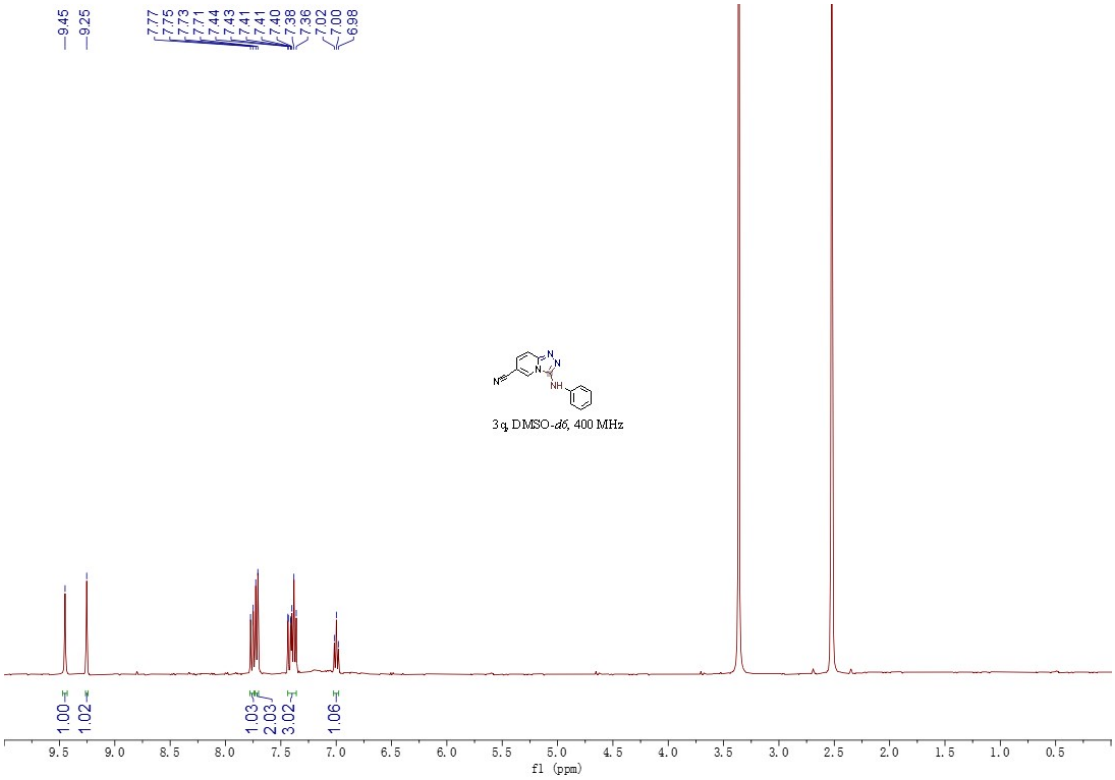
3m, DMSO-*d6*, 400 MHz

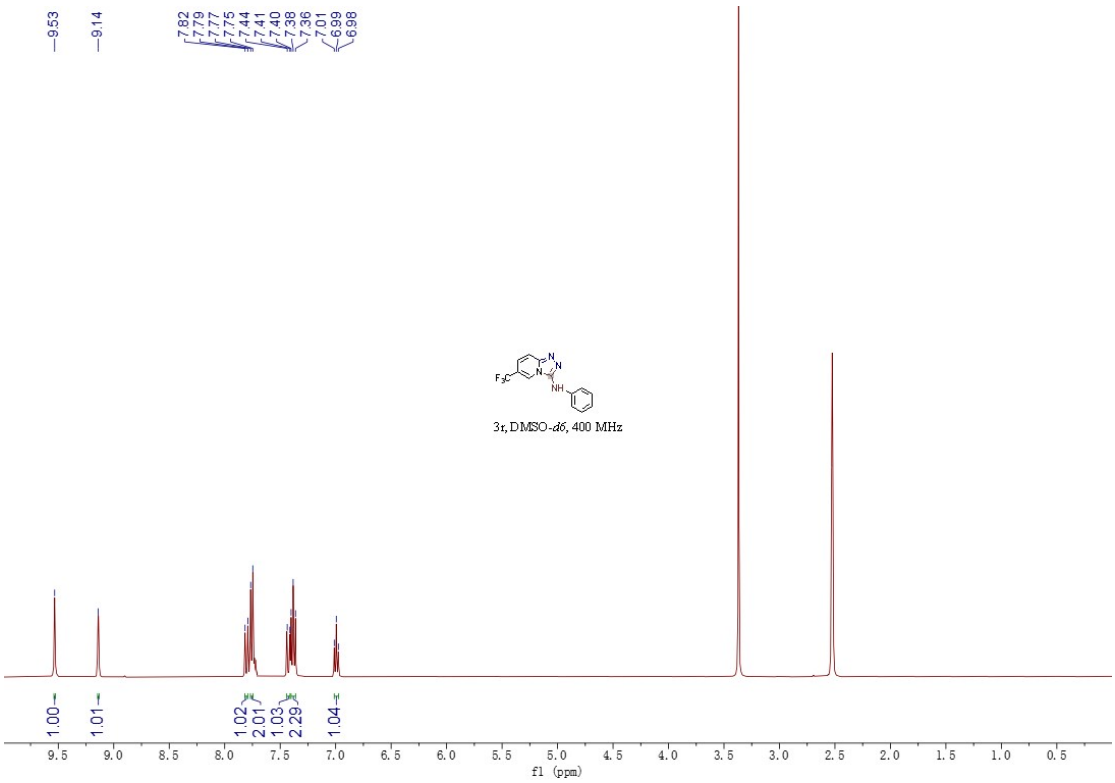


3n, DMSO-*d6*, 400 MHz


9.14
8.17
7.59
7.56
7.55
7.53
7.34
7.32
7.30
7.16
7.14
6.94
6.92
6.91
2.29
3o, DMSO-d6, 400 MHz
1.00
1.00
3.01
2.06
1.01
1.03
3.00
f1 (ppm)


9.29
8.72
7.69
7.66
7.38
7.36
7.34
7.30
7.29
7.27
7.27
6.98
6.97
6.95
3p, DMSO-d6, 400 MHz
1.00
1.00
3.01
2.01
1.02
1.01
f1 (ppm)


9.45
9.25
7.77
7.75
7.73
7.71
7.44
7.43
7.41
7.41
7.40
7.38
7.36
7.02
7.00
6.98
3q, DMSO-d6, 400 MHz
1.00
1.02
1.03
2.03
3.02
1.06
f1 (ppm)


9.53
9.14
7.82
7.79
7.77
7.75
7.44
7.41
7.40
7.38
7.36
7.01
6.99
6.98
3r, DMSO-d6, 400 MHz
1.00
1.01
1.02
2.01
1.03
2.29
1.04
f1 (ppm)

Reference

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2. Z. Liu, J. Huang, Y. Gu, et al., *JACS.*, 2022, **144**, 883-890.

3. K. Pandurangan, A. B. Aletti, D. Montroni, J. A. Kitchen, M. Martínez-Calvo, S. Blasco, T. Gunnlaugsson and E. M. Scanlan, *Org. Lett.*, 2017, **19**, 1068-1071.

4. (a) Y. Hu, L. Chen, C. Zou, J. He, L. Feng, J.-Q. Wu, W.-H. Chen and J. Hu, *Org. Lett.*, 2022, **24**, 5137-5142. (b) S. Jiao, Z. Wang, Q. Zhao, W. Yu and J. Chang, *Tetrahedron*, 2018, **74**, 3069-3073.