Supporting information for

Novel Furimazine Derivatives for NLuc Bioluminescence with Various C-6 and C-8 Substituents

Jie Li [a†], Xiaoxu Wang [a†], Gaopan Dong [a], Chongzheng Yan [a], Yuanyuan Cui [a], Zheng Zhang [a], Lupei Du [a], and Minyong Li [a*]

[a] Department of Medicinal Chemistry, Key Laboratory of Chemical Biology (MOE), School of Pharmaceutical Sciences, Cheeloo College of Medicine, Shandong University, Jinan, Shandong 250012, China.

* Corresponding Author: Tel/Fax: +86-531-8838-2076. Email: mli@sdu.edu.cn

[†]Authors contributed equally to this work.

CONTENT

Cytotoxicity of novel furimazine derivatives (CCK8)

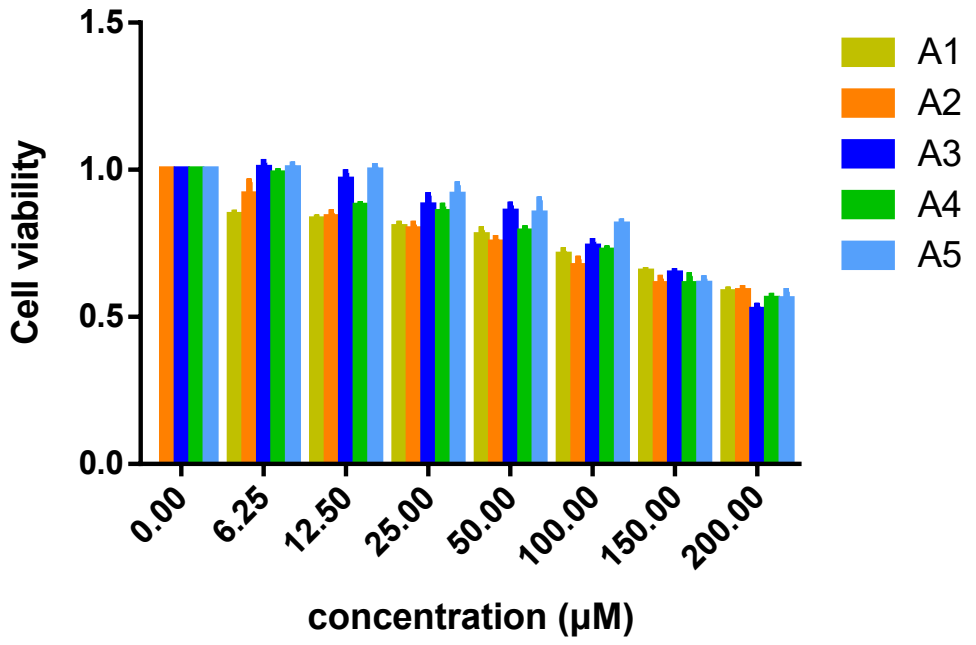


Figure S1. Cytotoxicity of novel furimazine derivatives (CCK8)

Preparation and quantitation of NanoLuc

When established the A549 cell lines expressing the NLuc, a flag-tag was combined with the gene sequence of NLuc. Thus, by the means of quantitation of flag-tag, the amount of NLuc in cells can be determined precisely.

A549 cells expressing NLuc (1 x 10^4) were diluted in 1 mL Tris-HCl (50 mM, pH 7.40). Through repeated freezing and thawing, the cells were destroyed, and the intracellular components were released. By centrifugation, the supernatant was collected. Then utilizing the flag-tag ELISA Kit (Shanghai Beyotime Biotechnology Co., Ltd.), the amount of NLuc was determined precisely, and drawn the standard curve y = 0.0134x + 0.0167. The concentration of supernatant containing NLuc was 10.5 μg/mL. The supernatant containing NLuc was frozen and stored at -80 °C before assay.

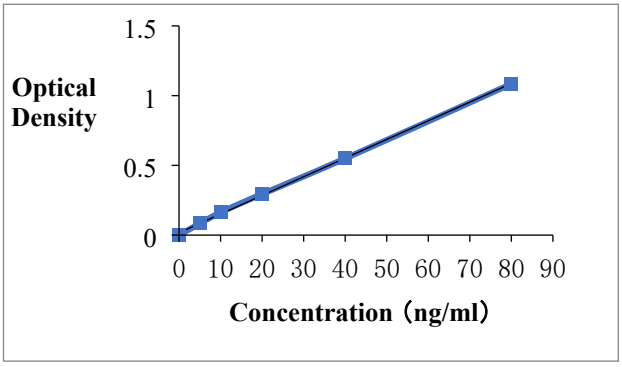
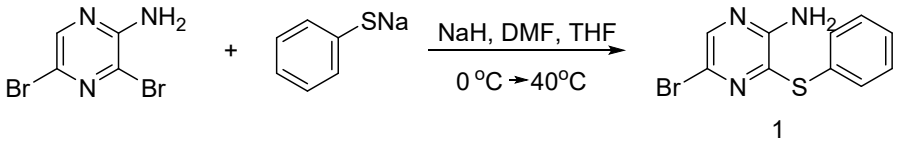


Figure S2. The standard curve between the optical density and flag-tag concentration.

Synthetic procedures

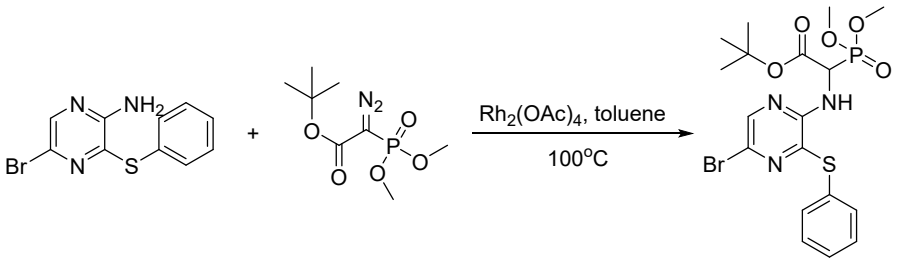
Synthesis of 5-bromo-3-(phenylthio) pyrazin-2-amine (1)



1

Sodium benzenethiolate (800 mg, 1.3 eq) was dissolved in dry DMF, and the mixture was cooled to 0 °C. NaH (4 eq) was slowly added to this solution, and the mixture was stirred at 0 °C for 1 h. Then add 3,5-dibromopyrazin-2-amine (11.17 g, 1.0 eq) dissolved in THF. The reaction mixture was continuously stirred at 40 °C for 1 h. The mixture was then filtered by Celatom, and the filtrate was poured in a saturated ammonium chloride solution extracted with ethyl acetate. After being dried over anhydrous sodium sulfate and concentrated under reduced pressure, the crude product was further purified by chromatography on silica gel (PE/EtOAc 5:1) to give a viscous yellow solid 560 mg, 43%). Melting point 94 °C- 96 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.97 (s, 1H), 7.48 – 7.40 (m, 5H), 6.73 (s, 2H). ESI-MS calcd. for $C_{10}H_8BrN_3S$ $(M+H)^+$:282.1, found: 282.36 .

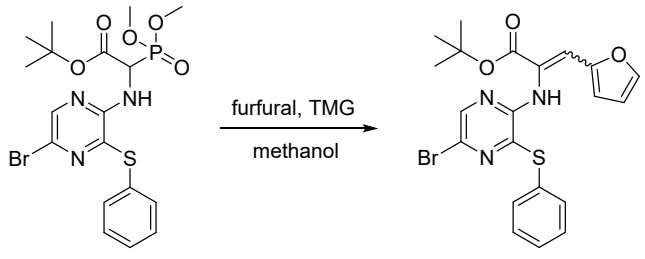
Synthesis of *tert*-butyl 2-((5-bromo-3-(phenylthio)pyrazin-2-yl)amino)-2-(dimethoxyphosphoryl) acetate (2)



1 2

In a 50-mL round bottom flask were placed 5-bromo-3-(phenylthio) pyrazin-2-a mine (360 mg, 1.27 mmol, 1.0eq), *tert*-butyl 2-diazo-2-(diethoxyphosphoryl) ace tate (760 mg, 2.54 mmol, 2.0 eq), $Rh_2(OAc)_4$ (56 mg,0.127 mmol, 0.1eq) and 20 mL toluene. The reaction was refluxed at 100 °C for 24 h and then allowe d to cool to room temperature; then the solution was extracted with ethyl acet ate and washed with saturated sodium chloride aqueous solution. After being d ried over anhydrous sodium sulfate and concentrated under reduced pressure, th e crude product was further purified by chromatography on silica gel (PE/EtO Ac 2:1) to give a dark brown solid (326 g, 48%). Melting point 145 °C- 147 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.20 (s, 1H), 7.54 – 7.39 (m, 5H), 6.22 (t, J = 6.6 Hz, 1H), 5.02 (dd, J = 22.0, 7.8 Hz, 1H), 3.79 – 3.64 (m, 8H), 1. 41 (s, 9H). ESI-MS calcd. $C_{18}H_{23}BrN_3O_5PS$ (M+H) $^+$: 504.1, found: 503.89

Synthesis of *tert*-butyl 2-((5-bromo-3-(phenylthio)pyrazin-2-yl)amino)-3-(furan-2-yl)acrylate (3)



2 3

In a 50-mL round bottom flask was placed *tert*-butyl 2-((5-bromo-3-(phenylthio) pyrazin-2-yl) amino)-2-(dimethoxyphosphoryl) acetate (326 mg, 0.61 mmol, 1.0eq), 2-furaldehyde (76 mg, 0.79 mmol, 1.3eq) and 20 mL methanol. Then TMG (140 mg, 1.22 mmol, 2.0 eq) was added to this solution, and the reaction was stirred at room temperature for 2 h. The reaction was poured in water and extracted with ethyl acetate.

After being dried over anhydrous sodium sulfate and concentrated under reduced pressure, the crude product was further purified by chromatography on silica gel (PE/EtOAc 10:1) to give a yellow solid (0.23 g, 79%). Melting point 112 °C-114 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.36 (s, 1H), 8.12 (s, 1H), 7.76 (s, 1H), 7.48 (dt, J = 17.4, 6.3 Hz, 5H), 7.06 (s, 1H), 6.73 (d, J = 3.1 Hz, 1H), 6.58 (s, 1H), 1.37 (s,9H). ESI-MS calcd. $C_{21}H_{20}BrN_3O_3S$ $(M+H)^+$: 474.3, found: 474

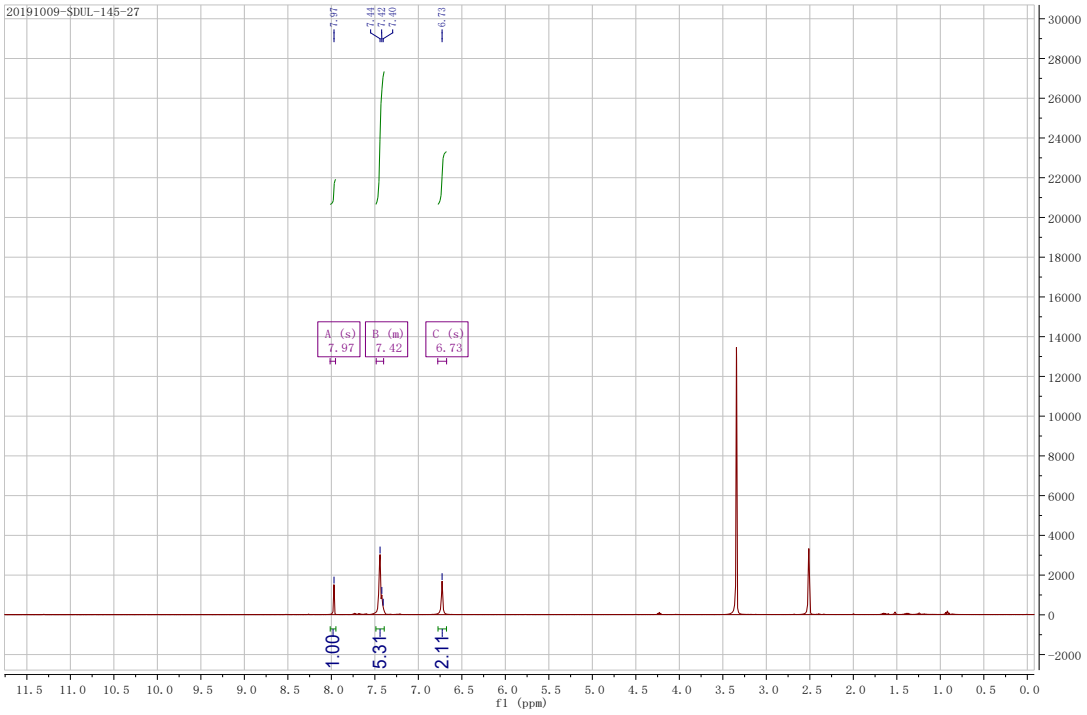
General procedure for preparation of furimazine analogs A1-A5.

Under the N_2 condition, *tert*-butyl 2-((5-bromo-3-(phenylthio) pyrazin-2-yl) amino)-3-(furan-2-yl) acrylate (200 mg, 0.422 mmol, 1.0 eq) was dissolved in 1,4-dioxane and H_2O. To this solution were added appropriate boronic acid or boronic acid pinacol ester (compounds A1, A2, A4 is boronic acid, compounds A3, A5 is boronic acid pinacol ester) (0.844 mmol, 2.0 eq), Pd $(PPh_3)_4$ (48.7 mg, 0.0438 mmol) and K_2CO_3 (165 mg, 1.26 mmol, 3.0 eq). The reaction mixture was heated to reflux at 85 °C for 3 h and then allowed to cool to room temperature. The reaction was poured in water and extracted with ethyl acetate. After being dried over anhydrous sodium sulfate and concentrated under reduced pressure, the crude product was further purified by chromatography on silica gel (PE/EtOAc 10:1) to give a yellow solid. To a solution of **4** (1 eq) in dichloromethane was added TFA (2 mL). The reaction mixture was stirred at room temperature for 4 h. Then all volatiles were removed under reduced pressure, and the residue was dried under a high vacuum. The crude product **5** didn't need further purification. The crude product **5** was dissolved in THF, and added the acetic anhydride (10 eq) and triethylamine (10 eq) cooled to 0 °C. Then, DMAP (0.1 eq) was added to this solution. 0.5 h later, the reactions removed to room temperature and poured in the water and extracted with dichloromethane, and dried over anhydrous Na_2SO4. The crude product was further purified by chromatography on silica gel using dichloromethane as eluent. The corresponding dehydrocoelenterazine with the general structure **6** was isolated as red solid and used in the next step without further purifications. Dehydrocoelenterazine **6** was dissolved in dichloromethane and methanol then cooled to 0 °C. $NaBH_4$ (4 eq) was added to this solution, and the mixture was

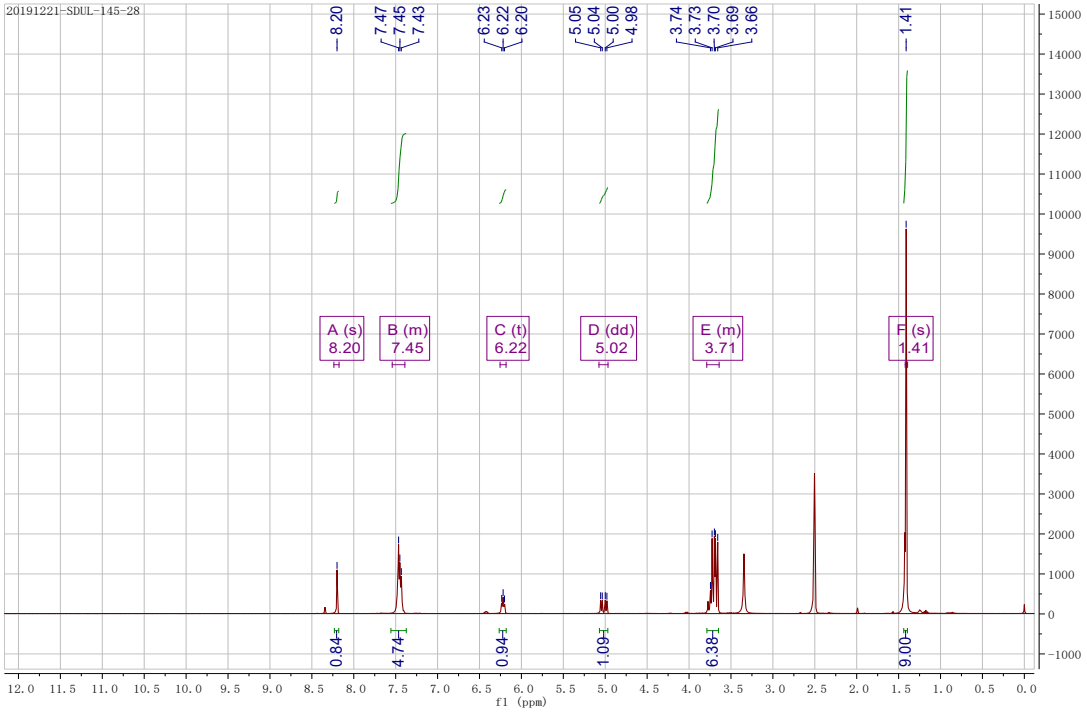
stirred at 0 ºC for 0.5 h. The reaction mixture was quenched with 0.1 M HCl and extracted with dichloromethane, and dried over anhydrous Na_2SO4. The crude was concentrated under vacuum and further purified by chromatography on silica gel (DCM/MeOH 30:1). The target furimazine analog was isolated pure as a yellow solid and dried on high vacuum.

NMR, ESI-HRMS, and HPLC spectra

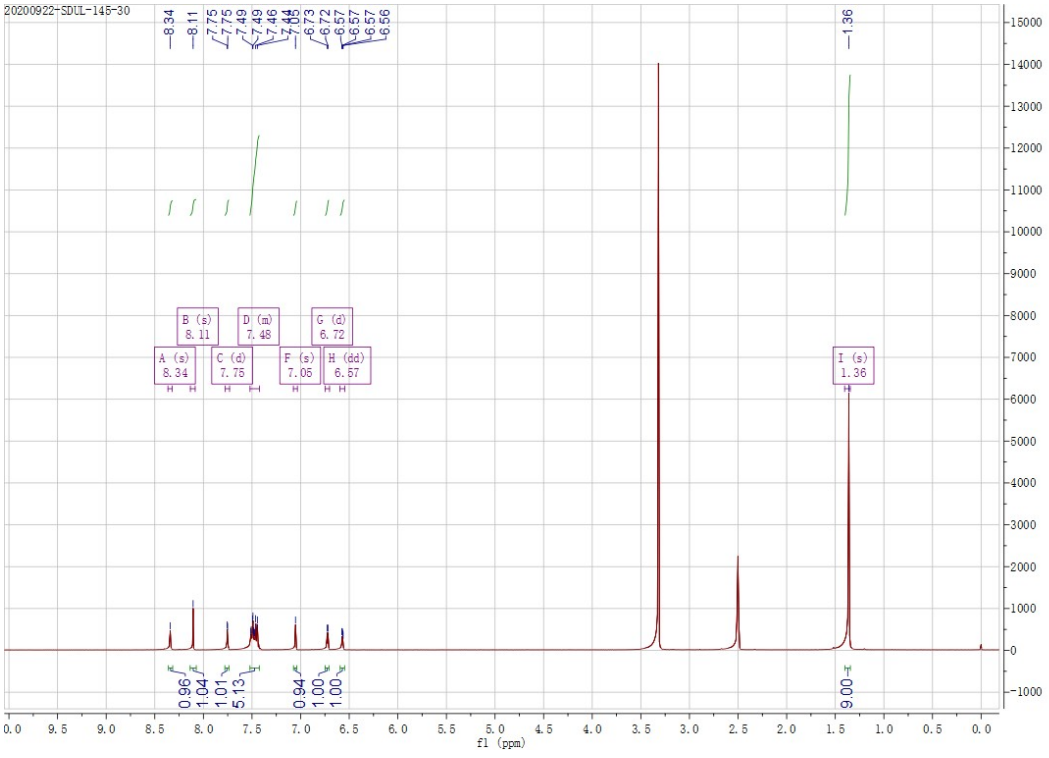
5-bromo-3-(phenylthio) pyrazin-2-amine (1)



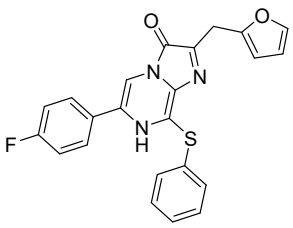
tert-butyl 2-((5-bromo-3-(phenylthio) pyrazin-2-yl) amino)-2-(dimethoxyphosphoryl) acetate (2)



tert-butyl 2-((5-bromo-3-(phenylthio) pyrazin-2-yl) amino)-3-(furan-2-yl)acrylate (3)



6-(4-fluorophenyl)-2-(furan-2-ylmethyl)-8-(phenylthio)imidazo[1,2-a]pyrazin-3(7H)-one (A1)



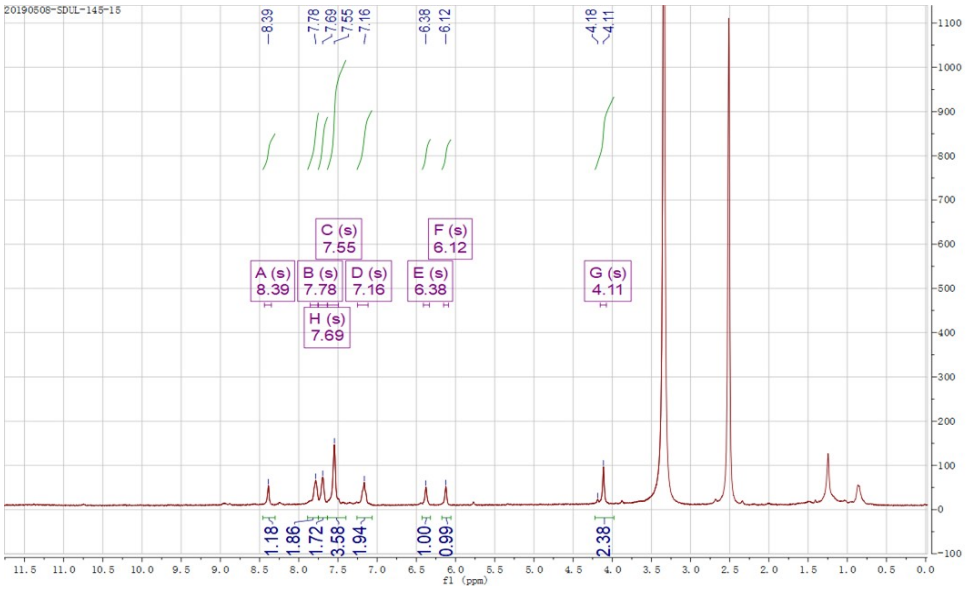
Isolated pure as yellow solid. Yield 42%. Melting point 112 ^{o}C- 115 ^{o}C. HPLC purity 99.7%

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.39 (s, 1H), 7.78 (s, 2H), 7.69 (s, 2H), 7.55 (s, 4H), 7.16 (s, 2H), 6.38 (s, 1H), 6.12 (s, 1H), 4.11 (s, 2H).

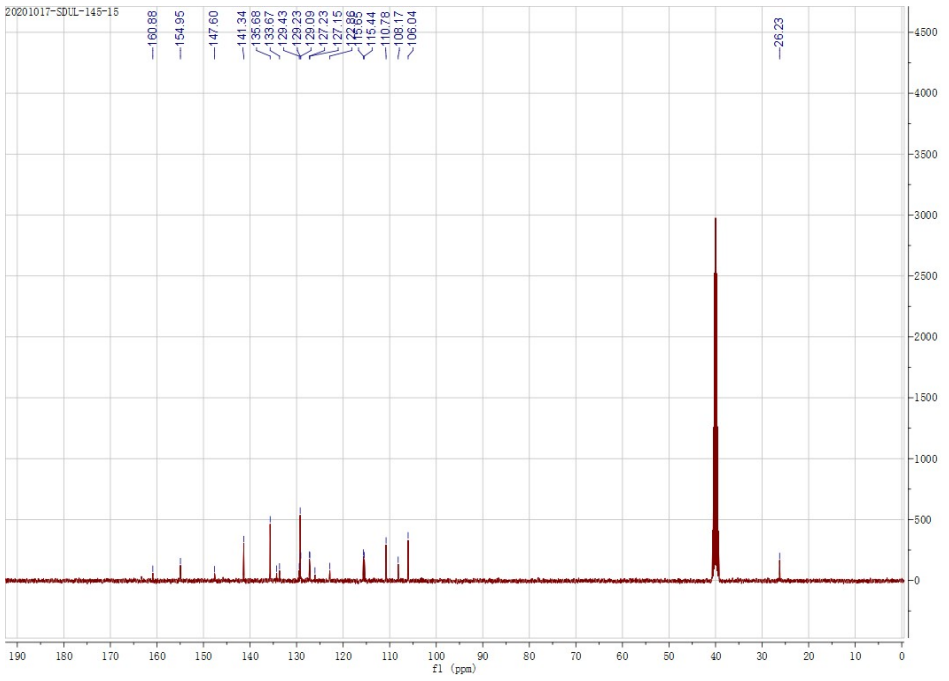
^{13}C NMR (400 MHz, DMSO-d_6) δ 160.88, 154.95, 147.60, 141.34, 135.68, 134.28, 133.67, 129.43, 129.23, 129.09, 127.23, 127.15, 126.05, 122.86, 115.65, 115.44, 110.78, 108.17, 106.04, 26.23.

ESI-HRMS calcd. $C_{23}H_{16}FN_3O_2S$: 417.0947, found:418.1017 $(M+H)^+$.

^{1}H NMR



^{13}C NMR



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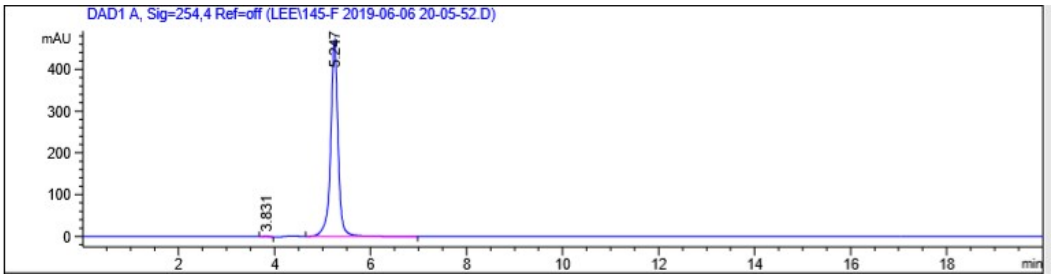
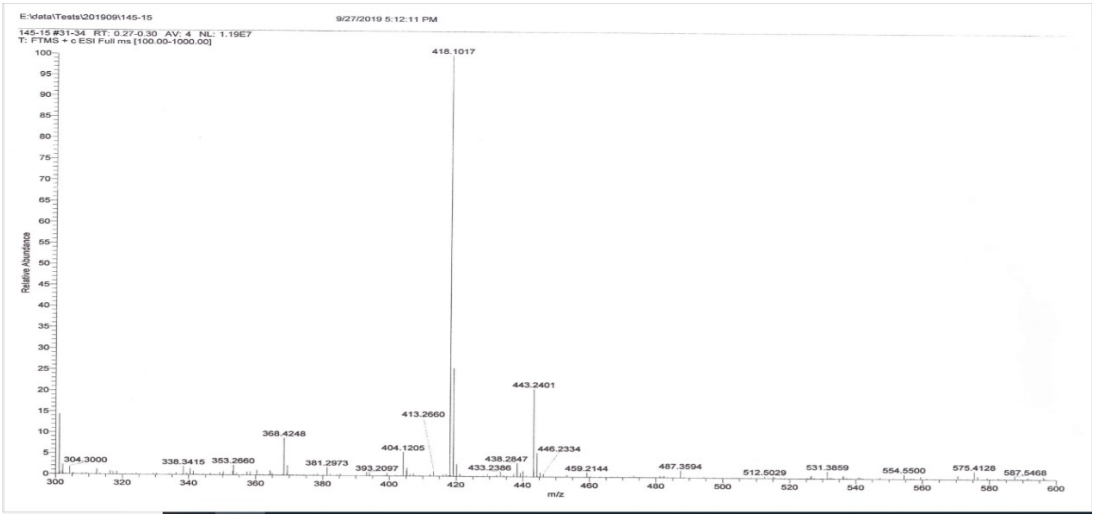
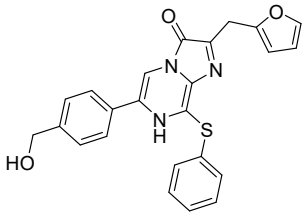


ESI-HRMS



2-(furan-2-ylmethyl)-6-(4-(hydroxymethyl)phenyl)-8-(phenylthio)imidazo[1,2-a]pyrazin-3(7H)-one (A2)



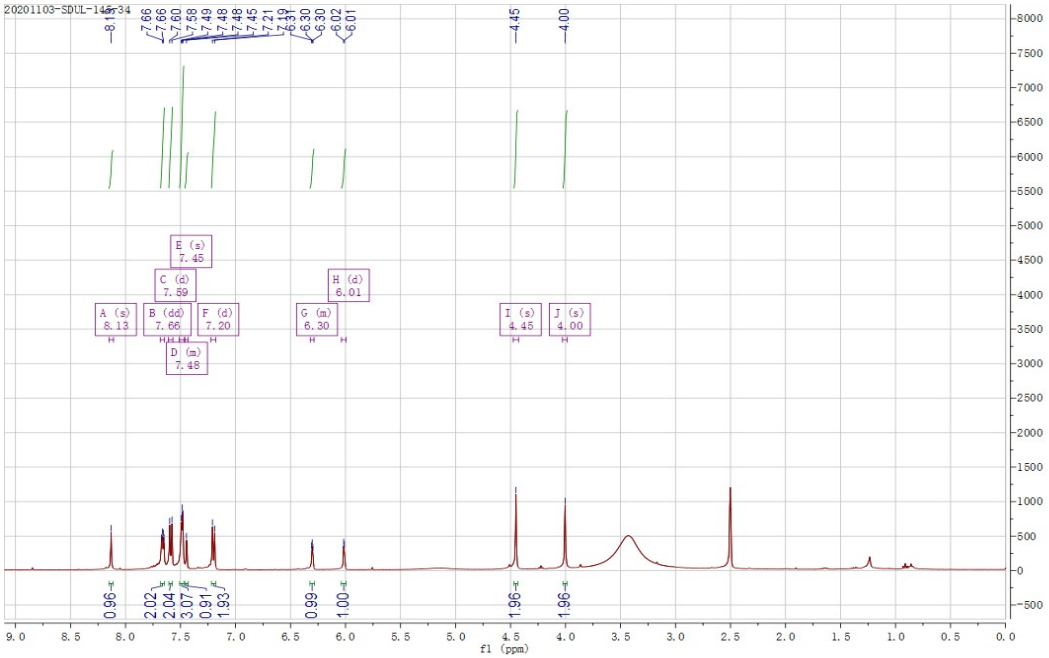
Isolated pure as yellow solid. Yield 42%. Melting point 150 ^{o}C- 157 ^{o}C. HPLC purity 99.9%

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.13 (s, 1H), 7.66 (dd, J = 6.5, 3.0 Hz, 2H), 7.59 (d, J = 8.2 Hz, 2H), 7.51 – 7.46 (m, 3H), 7.45 (s, 1H), 7.20 (d, J = 8.2 Hz, 2H), 6.32 – 6.29 (m, 1H), 6.01 (d, J = 2.6 Hz, 1H), 4.45 (s, 2H), 4.00 (s, 2H).

^{13}C NMR (400 MHz, DMSO-d_6) δ 154.67, 148.08, 142.29, 141.43, 135.98, 135.86, 134.91, 129.26, 129.18, 126.97, 126.67, 125.13, 123.03, 110.79, 108.04, 106.13, 63.11, 26.14.

ESI-HRMS calcd. for $C_{24}H_{19}N_3O_3S$: 429.1147, found: 430.1222 $(M+H)^+$.

^{1}H NMR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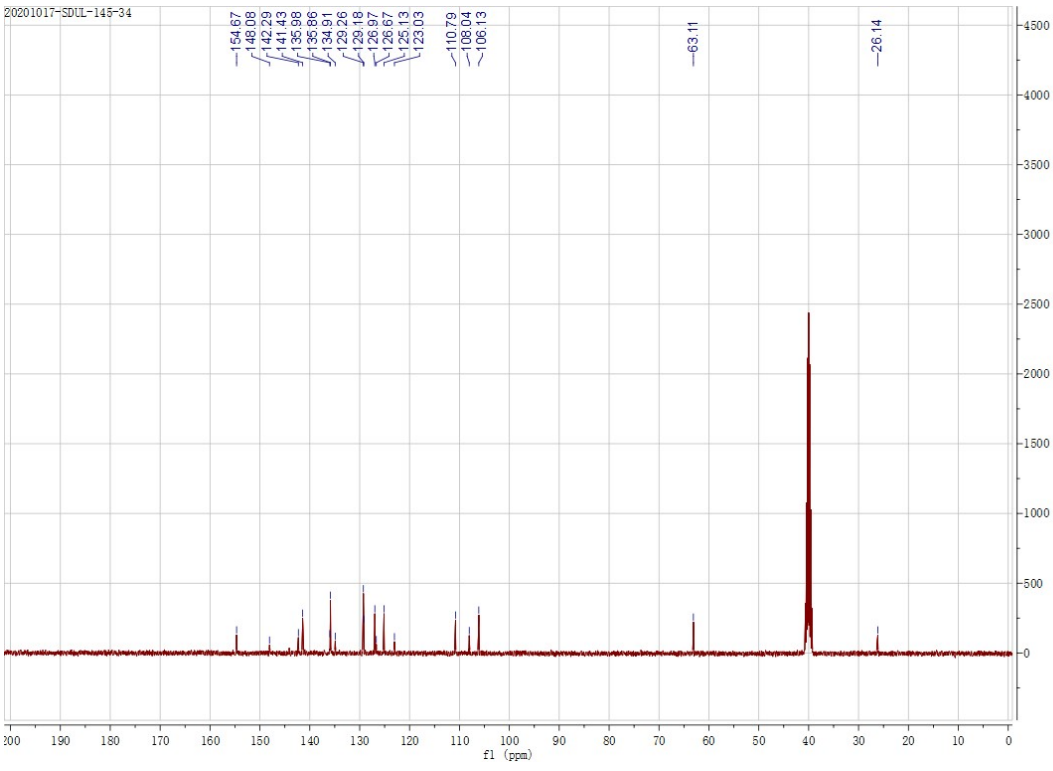
C (d) 7.59
H (d) 6.01
A (s) 8.13
B (dd) 7.66
F (d) 7.20
G (m) 6.30
I (s) 4.45
J (s) 4.00
D (m) 7.48
f1 (ppm)

^{13}C NMR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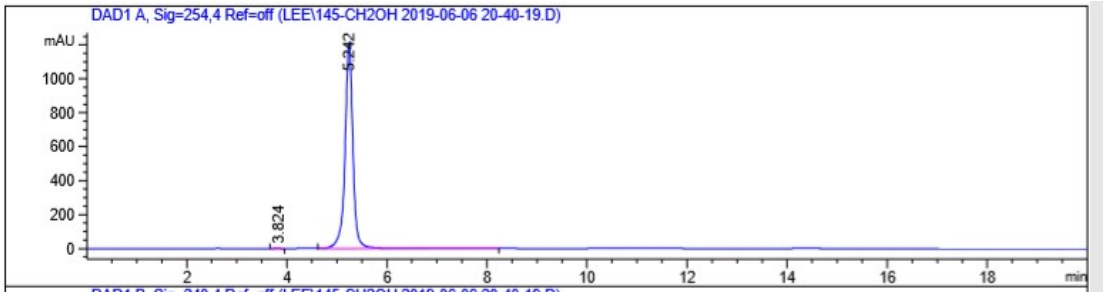
HPLC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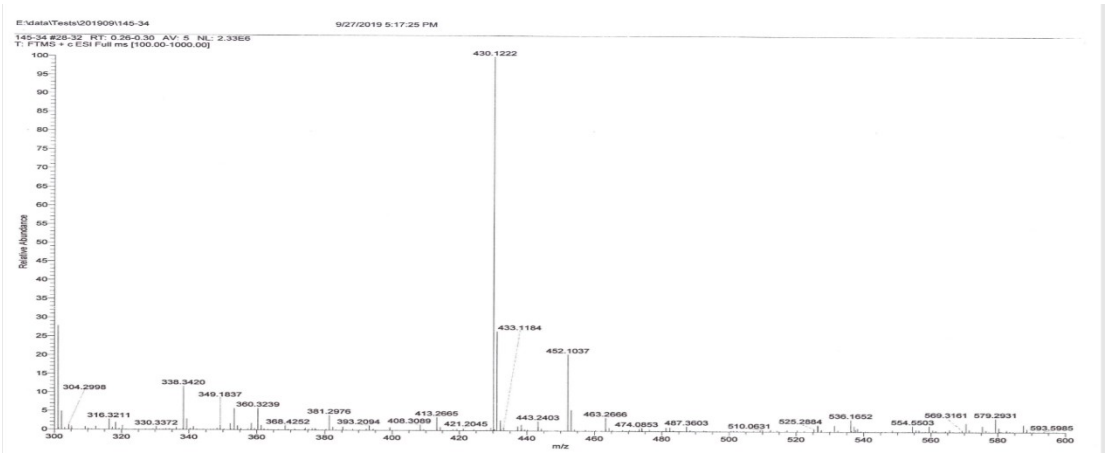
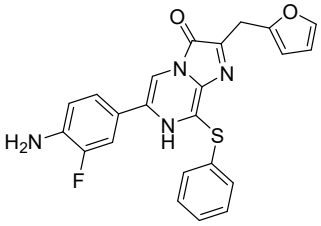


6-(4-amino-3-fluorophenyl)-2-(furan-2-ylmethyl)-8-(phenylthio)imidazo[1,2-a]pyrazin-3(7H)-one (A3)



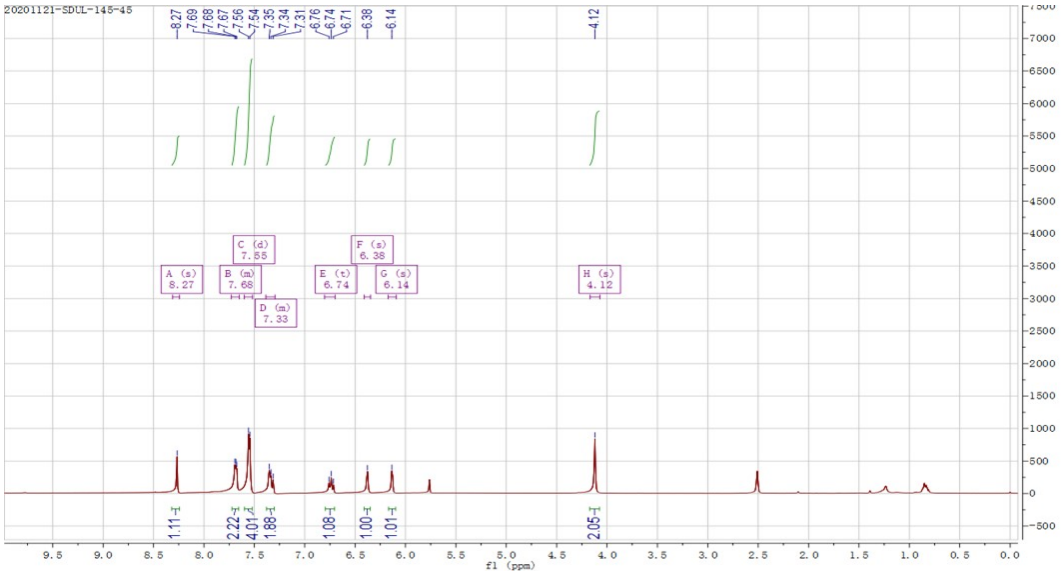
Isolated pure as yellow solid. Yield 42%. Melting point 135 ^{o}C- 145 ^{o}C. HPLC purity 99.8%

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.27 (s, 1H), 7.73 – 7.65 (m, 2H), 7.55 (d, J = 5.2 Hz, 4H), 7.39 – 7.29 (m, 2H), 6.74 (t, J = 8.9 Hz, 1H), 6.38 (s, 1H), 6.14 (s, 1H), 4.12 (s, 2H).

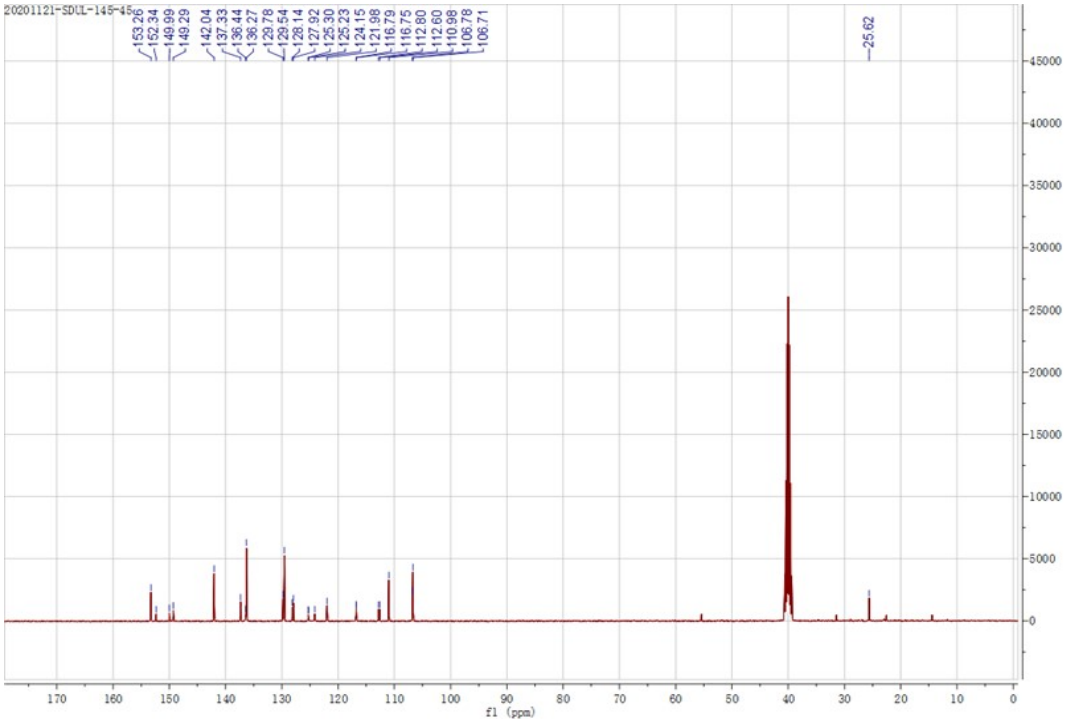
^{13}C NMR (400 MHz, DMSO-d_6) δ 153.26, 152.34, 149.99, 149.29, 142.04, 137.33, 136.44, 136.27, 129.78, 129.54, 128.14, 127.92, 125.30, 125.23, 124.15, 121.98, 116.79, 116.75, 112.80, 112.60, 110.98, 106.78, 106.71, 25.62.

ESI-HRMS calcd. for $C_{23}H_{17}FN_4O_2S$: 432.1056, found: 433.1126 $(M+H)^+$.

^{1}H NMR



^{13}C NMR



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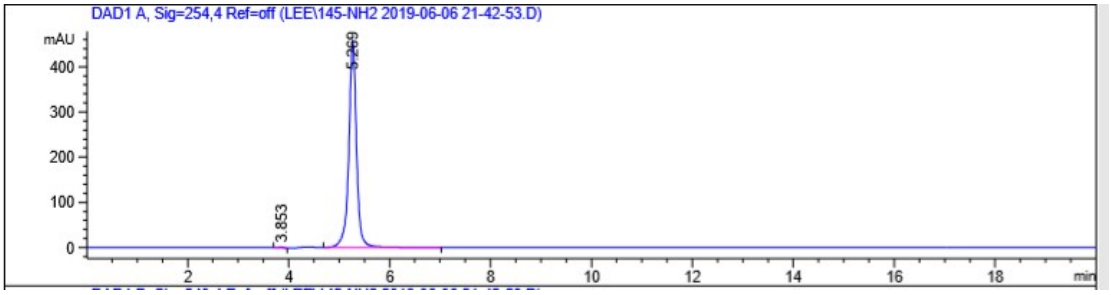
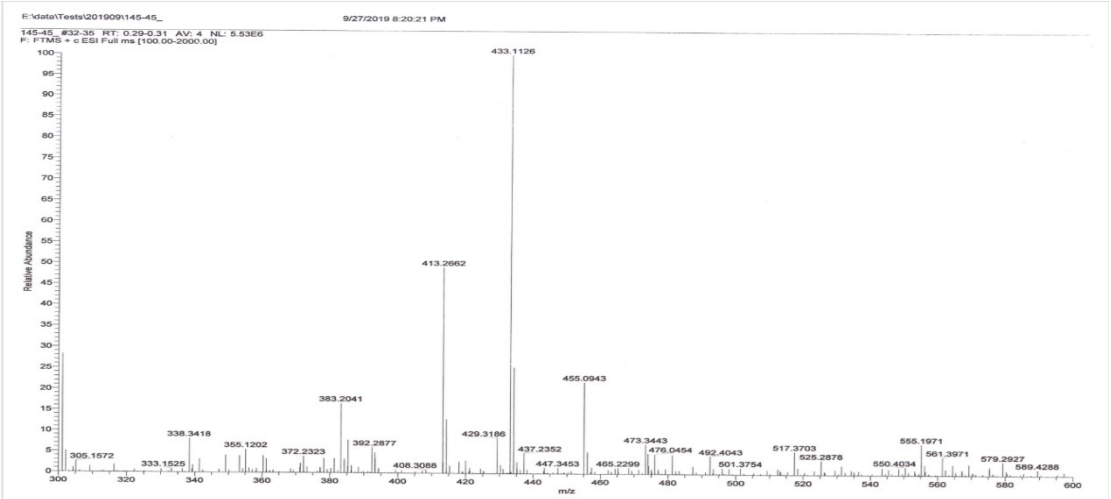
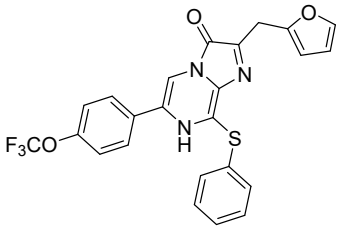


ESI-HRMS



2-(furan-2-ylmethyl)-8-(phenylthio)-6-(4-(trifluoromethoxy)phenyl)imidazo[1,2-a]pyrazin-3(7H)-one (A4)



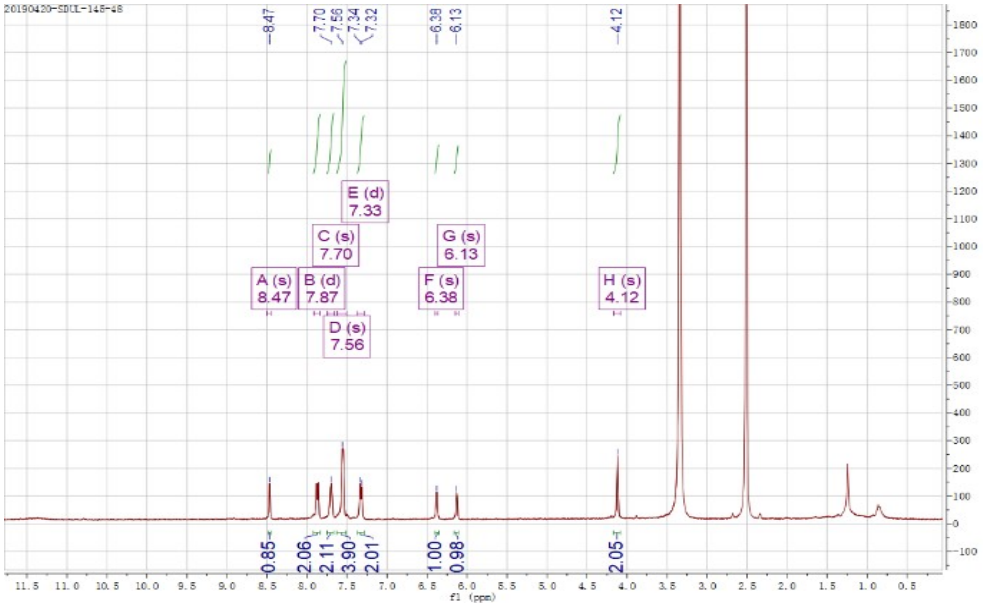
Isolated pure as yellow solid. Yield 42%. Melting point 180 ^{o}C- 185 ^{o}C. HPLC purity 99.7%

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.47 (s, 1H), 7.87 (d, J = 8.2 Hz, 2H), 7.70 (s, 2H), 7.56 (s, 4H), 7.33 (d, J = 8.4 Hz, 2H), 6.38 (s, 1H), 6.13 (s, 1H), 4.12 (s, 2H).

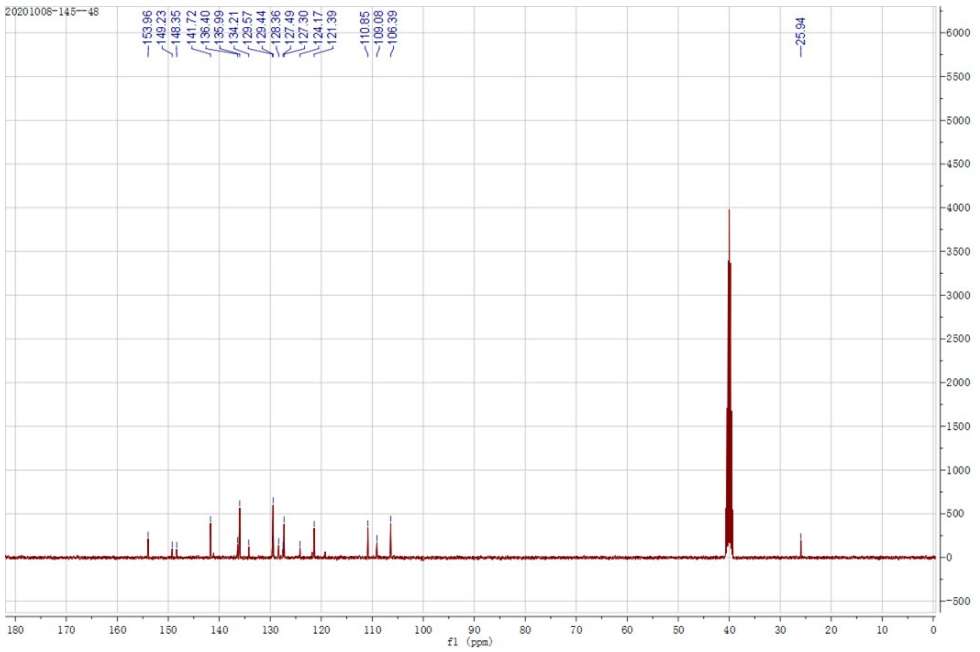
^{13}C NMR (400 MHz, DMSO-d_6) δ 153.96, 149.23, 148.35, 141.72, 136.40, 135.99, 134.21, 129.57, 129.44, 128.36, 127.49, 127.30, 124.17, 121.39, 110.85, 109.08, 106.39, 25.94.

ESI-HRMS calcd. for $C_{24}H_{16}F_3N_3O_3S$: 483.0864, found: 484.0938 $(M+H)^+$.

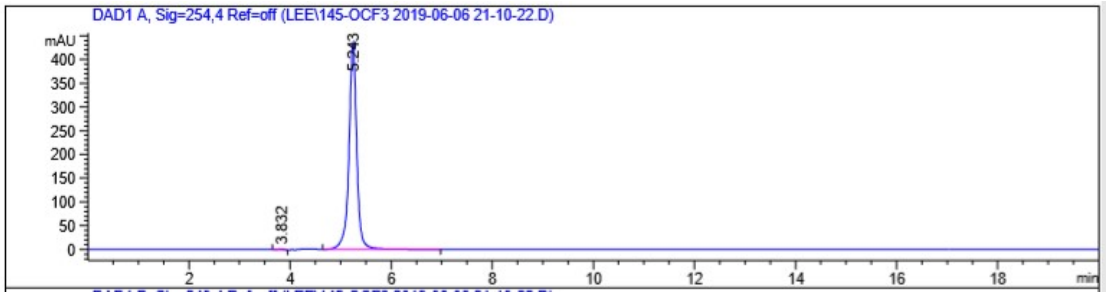
^{1}H NMR



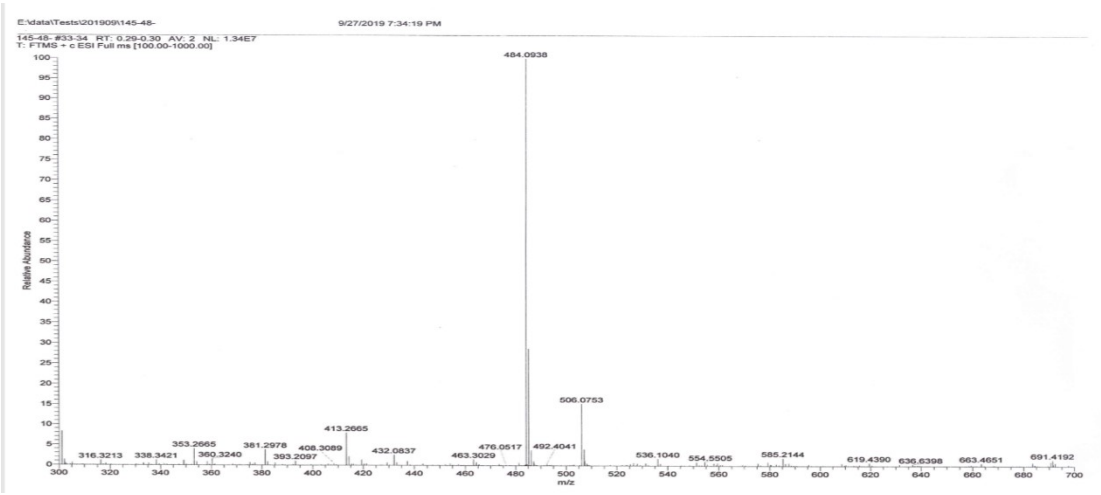
^{13}C NMR



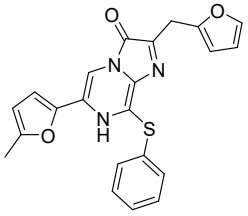
HPLC



ESI-HRMS



2-(furan-2-ylmethyl)-6-(5-methylfuran-2-yl)-8-(phenylthio)imidazo[1,2-a]pyrazin-3(7H)-one (A5)



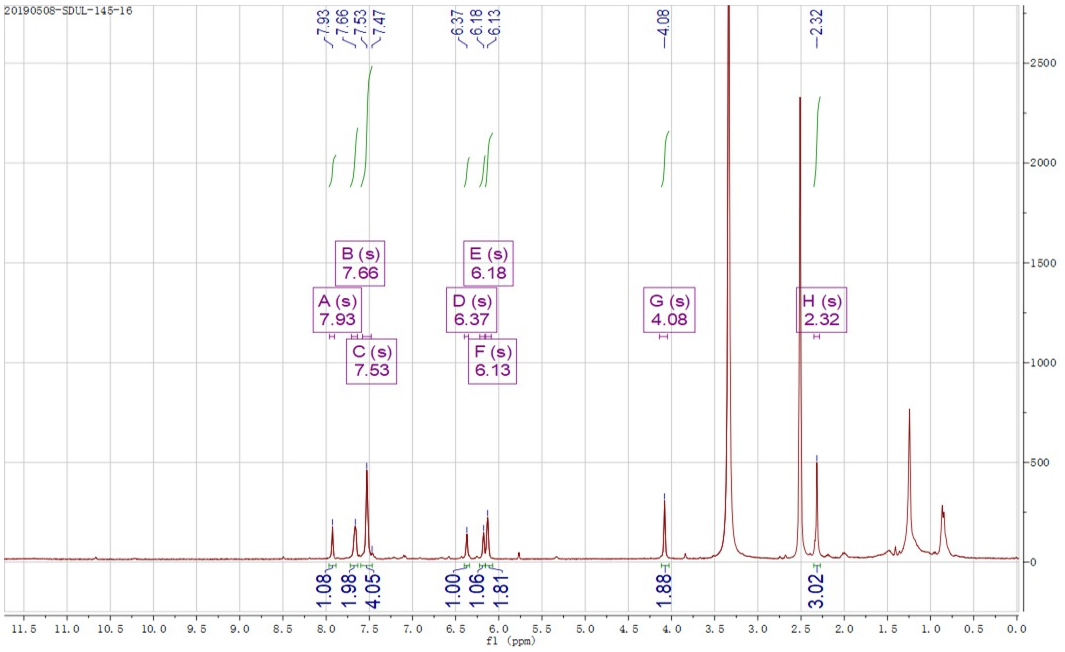
Isolated pure as yellow solid. Yield 42%. Melting point 108 ^{o}C-115 ^{o}C. HPLC purity 99.7%.

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.93 (s, 1H), 7.66 (s, 2H), 7.53 (s, 4H), 6.37 (s, 1H), 6.18 (s, 1H), 6.13 (s, 2H), 4.08 (s, 2H), 2.32 (s, 3H).

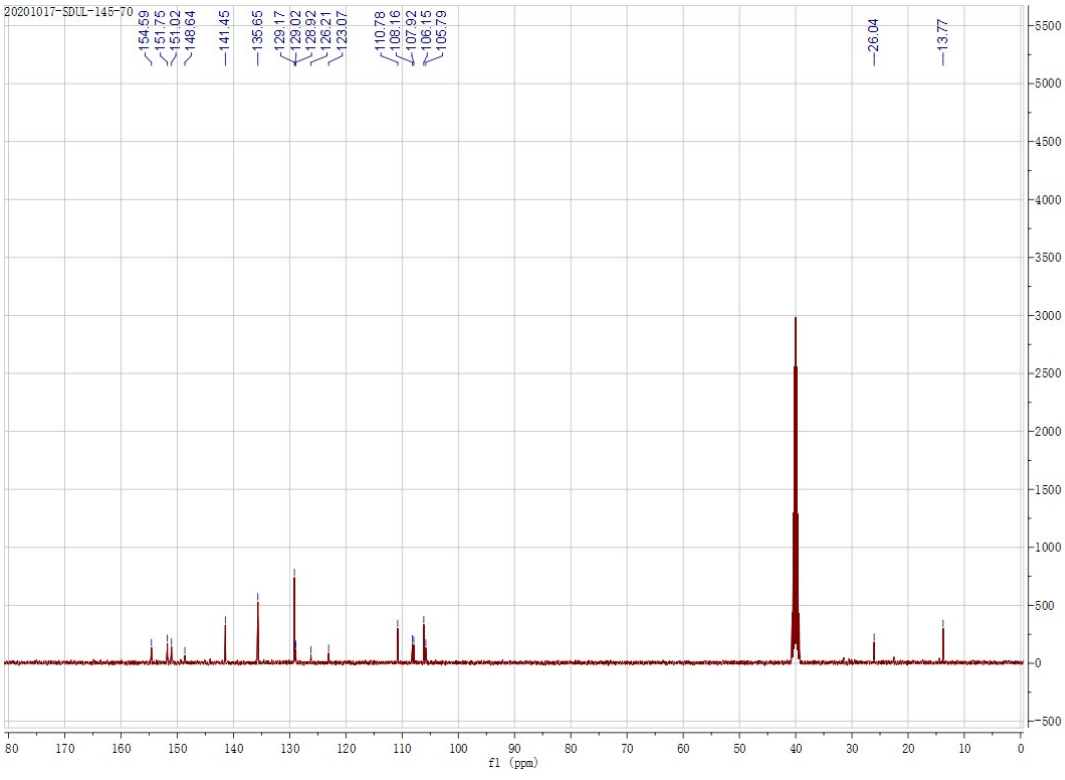
^{13}C NMR (400 MHz, DMSO-d_6) δ 154.59, 151.75, 151.02, 148.64, 141.45, 135.65, 129.17, 129.02, 128.92, 126.21, 123.07, 110.78, 108.16, 107.92, 106.15, 105.79, 26.04, 13.77.

ESI-HRMS calcd. for $C_{22}H_{17}N_3O_3S$: 403.0991, found: 404.1064$(M+H)^+$.

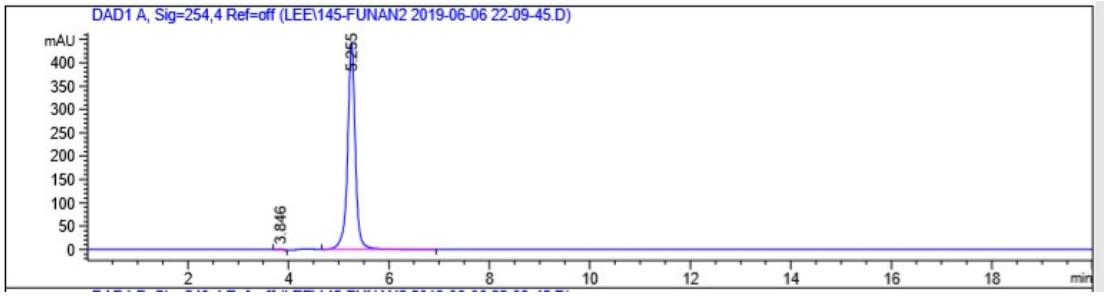
^{1}H NMR


20190508-SDUL-145-16
7.93
7.66
7.53
7.47
6.37
6.18
6.13
4.08
2.32
B (s) 7.66
A (s) 7.93
C (s) 7.53
E (s) 6.18
D (s) 6.37
F (s) 6.13
G (s) 4.08
H (s) 2.32
1.08
1.98
4.05
1.00
1.06
1.81
1.88
3.02
f1 (ppm)

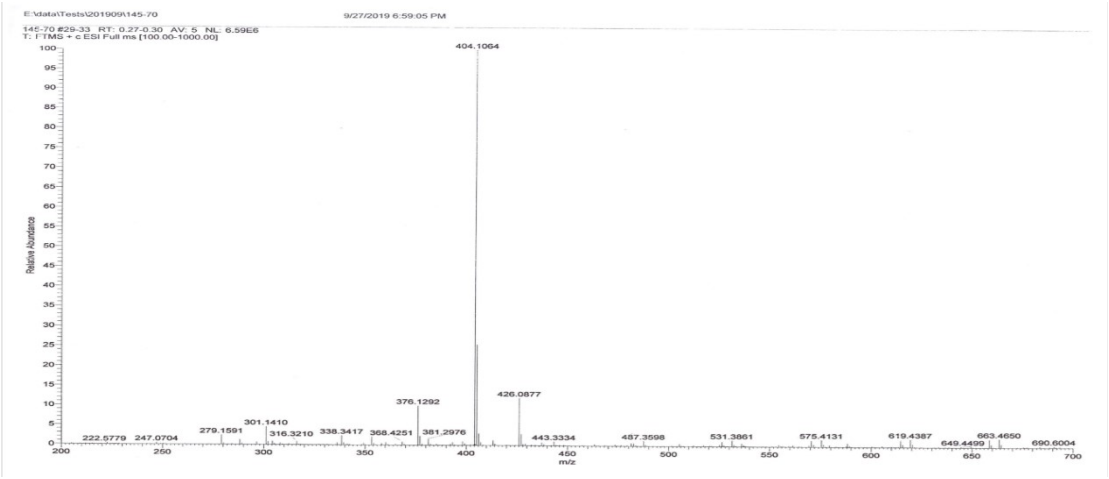
^{13}C NMR


20201017-SDUL-145-70
154.59
151.75
151.02
148.64
141.45
135.65
129.17
129.02
128.92
126.21
123.07
110.78
108.16
107.92
106.15
105.79
26.04
13.77
f1 (ppm)

HPLC


DAD1 A, Sig=254,4 Ref=off (LEE\145-FUNAN2 2019-06-06 22-09-45.D)
mAU
5.255
3.846
min

ESI-HRMS


E:\data\Tests\201909\145-70
9/27/2019 6:59:05 PM
145-70 #29-33 RT: 0.27-0.30 AV: 5 NL: 6.59E6
T: FTMS + c ESI Full ms [100.00-1000.00]
Relative Abundance
m/z
404.1064
426.0877
376.1292
301.1410
279.1591
316.3210
338.3417
368.4251
381.2976
222.5779
247.0704
443.3334
487.3598
531.3861
575.4131
619.4387
649.4499
663.4650
690.6004