SGC-CLK-1: a chemical probe for the Cdc2-Like kinases CLK1, CLK2, and CLK4

Deanna Tiek[1], Carrow I. Wells[2], Martin Schröder[4,5], Xiao Song[1], Carla Alamillo-Ferrer[2] Anshika Goenka[1], Rebeca Iglesia[1], Minghui Lu[1], Bo Hu[1], Frank Kwarcinski[3], Parvathi Sintha[3], Chandi de Silva[3], Mohammad Anwar Hossain[2], Alfredo Picado[2], William Zuercher[2], Reena Zutshi[3], Stefan Knapp[4,5], Rebecca B. Riggins[6], Shi-Yuan Cheng[1], and David H. Drewry[2,7]*

[1]The Ken & Ruth Davee Department of Neurology, The Lou and Jean Malnati Brain Tumor Institute, The Robert H. Lurie Comprehensive Cancer Center, Simpson Querrey Institute for Epigenetics, Northwestern University Feinberg School of Medicine, Chicago, IL 60611, USA.

[2]Structural Genomics Consortium, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599, USA

[3]Luceome Biotechnologies LLC, Tucson, AZ 85719 USA

[4]Structural Genomics Consortium (SGC), Buchmann Institute for Life Sciences (BMLS), Goethe University Frankfurt am Main, Max-von-Laue-Str. 15, 60438 Frankfurt am Main, Germany

[5]Institut für Pharmazeutische Chemie, Goethe University Frankfurt am Main, Max-von-Laue-Str. 9, Frankfurt am Main 60438, Germany.

[6]Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington DC 20057, USA

[7]UNC Lineberger Comprehensive Cancer Center, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599, USA

Material available in this supplemental data document:

Table S1: SGC-CLK-1 Activity Profile

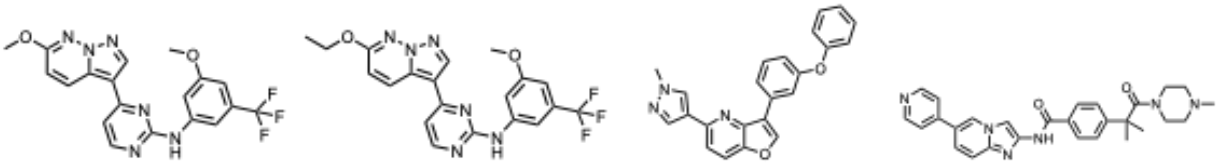
Kinase	PoC values (percent of control) (at 1µM inhibitor) SGC-CLK-1	Enzyme IC_{50} (nM)	NanoBRET (nM)
CLK1	8	13	165
CLK2	**16**	**4**	**70**
CLK3	92	363	nt**
CLK4	13	46	100
HIPK1	31	50	>10,000
HIPK2	34	42	>10,000
NEK7	40	nt	nt**
PIP5K2B	50	nt	nt**
STK16	53	49	167

Table S2: SGC-CLK-1N Enzymatic Profile

Eurofins Enzymatic Screening

Kinase	SGC-CLK-1N (CAF-225) IC_{50} (nM)
CLK1	>10,000
CLK2	>10,000
CLK3	>10,000
CLK4	>10,000
DYRK1A	>10,000
DYRK1B	>10,000
DYRK2	>10,000
DYRK3	>10,000
HIPK1	>10,000
HIPK2	>10,000
HIPK3	>10,000
HIPK4	>10,000
Pim-2	>10,000

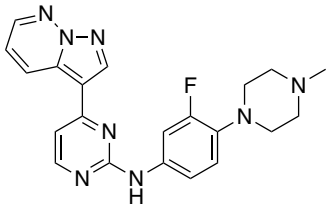
Table S3: Activity profile of the reported CLK inhibitors and the CLK probe (SGC-CLK-1)



	SGC-CLK-1	CAF-022	VN412 (12m)	CLK-T3
CLK1 (Enzyme)	13 nM	10 nM		0.67 nM
CLK2 (Enzyme)	4 nM	0.73 nM	10 nM	15 nM
CLK3 (Enzyme)	363 nM	no data		110 nM
CLK4	46 nM	8.8 nM		no data
CLK1 (NB)	154	10,000 nM		4 nM
CLK2 (NB)	58	580 nM		17 nM
CLK3 (NB)	NT	no data		no data
CLK4 (NB)	137	690 nM		2 nM
PubMed ID	This manuscript	35731869	30569600	28232751

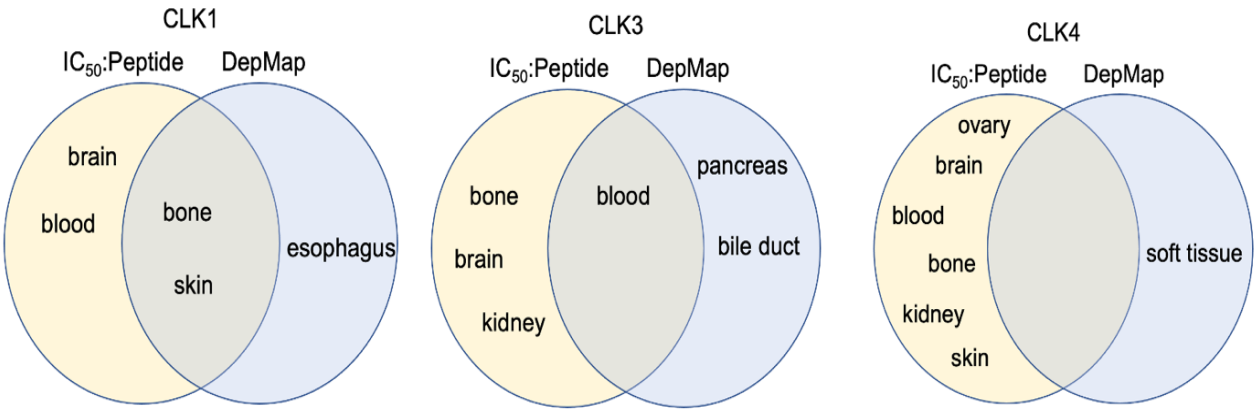
Table S3: "Enzyme" data refers to IC50 values generated from an assay that measures the inhibition of phosphorylation of a substrate (generated at Eurofins). "NB" data refers to the NanoBRET in cell target engagement assay as described in the body of the paper. Because it measures binding to the kinase in question in a cell, there can be a substantial drop-off in potency from an enzyme assay to a cellular readout. The differences seen can be compound series and target dependent. Reasons for this vary and can include differential uptake of compound into the cell.

The structure of CAF-052 (compound in pdb code 7ak3) is here:



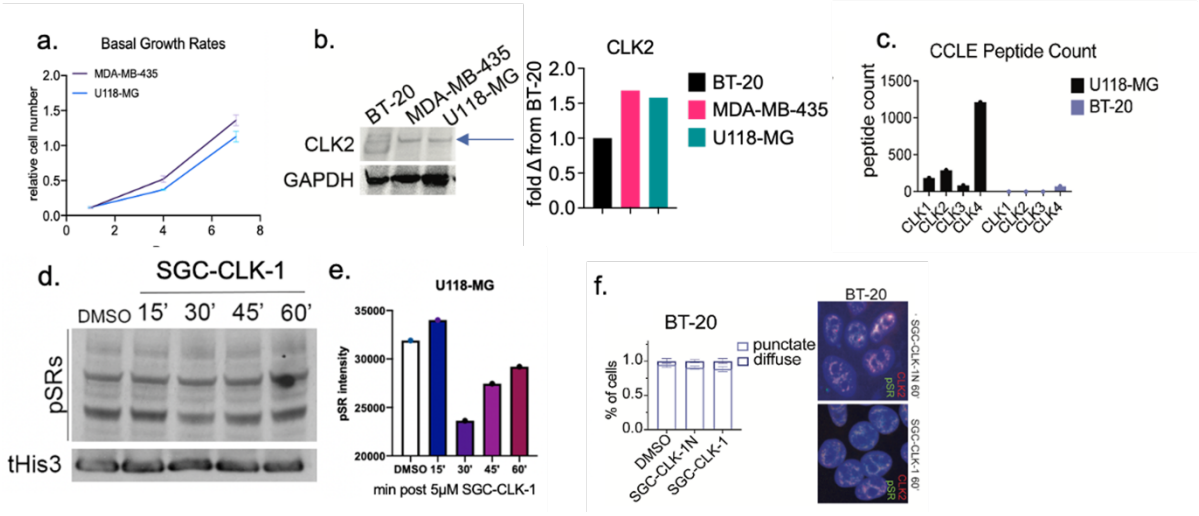
No data is show for CAF-052 in Table S3, as we did not pursue the same detailed CLK family characterization because it is significantly less selective (unpublished data) than the analogs we chose to pursue.

Figure S1: Venn Diagrams of overlap between DepMap and CL100 screen [Related to Fig 5]



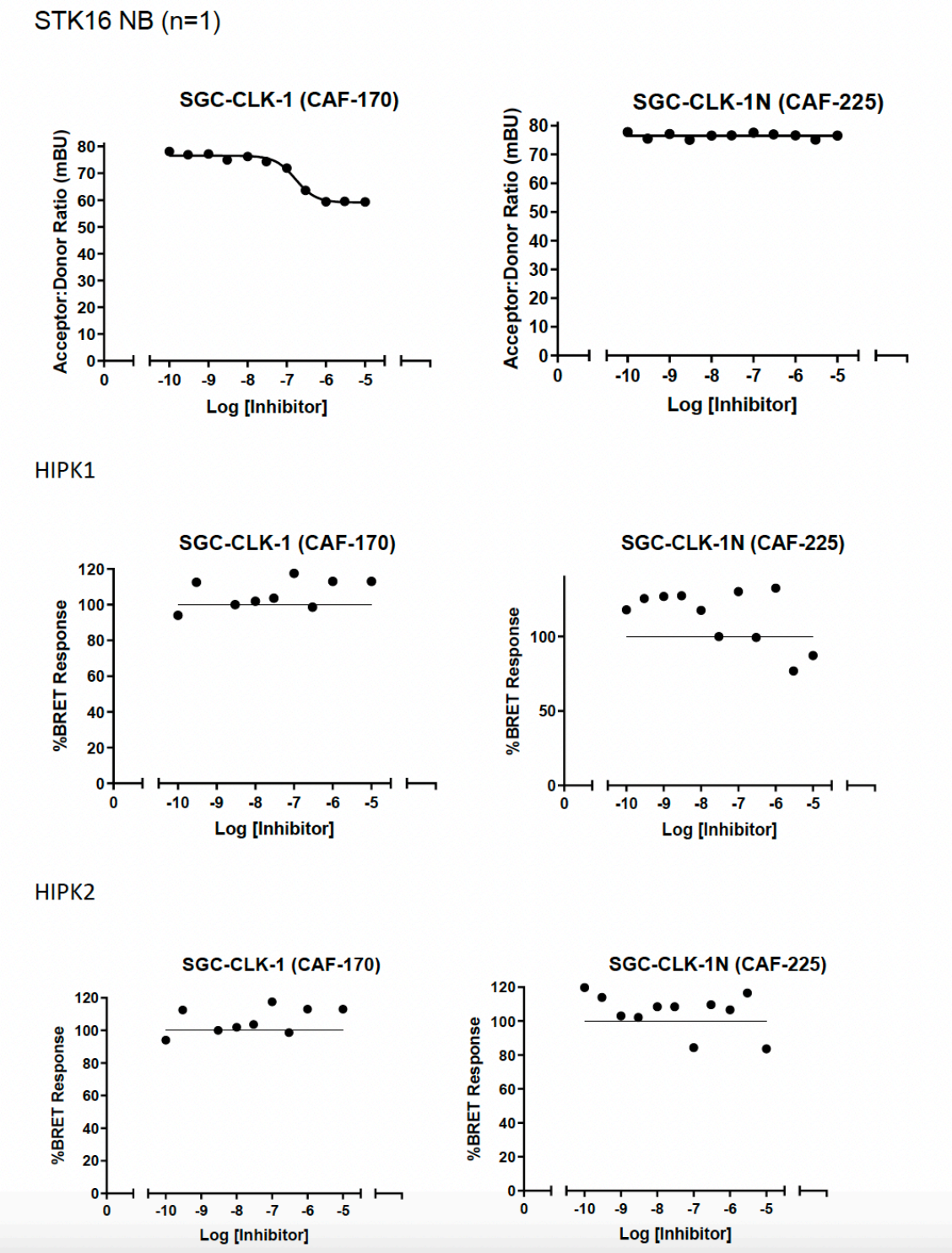
Supplementary Figure 1. Venn Diagrams of CLK1,3 and 4 overlap between DepMap and CL100 screen. IC50:Peptide and DepMap Venn Diagrams for the other 3 CLK members, where CLK2 has the highest overlap of predicted dependencies.

Figure S2. Changes in phosphorylation with high dose SGC-CLK-1 [Related to Fig 6]



Supplementary Figure 2. Changes in phosphorylation with high dose SGC-CLK-1. A. Crystal violet assay to determine basal growth rates of denoted cells at denoted time points. B. Western blot of CLK2 in denoted cell lines and quantification depicted in bar chart. C. CCLE peptide counts for CCLE lines U118-MG and BT-20 (MDA-MB-435 not included). D. Western blot of pSR proteins (1H4) in U118-MG cells post 5μM CAF-170 at denoted times (left), with E. quantification on the right. F. CLK2 distribution with 5 μM SGC-CLK-1N (CAF-225), SGC-CLK-1 (CAF-170) or DMSO with representative images on the right.

Figure S3: Off-target activity of SGC-CLK-1 and SGC-CLK-1N [Related to Table 2]



Supplementary Figure S3: Compound characterization in NanoBRET (NB) assays.

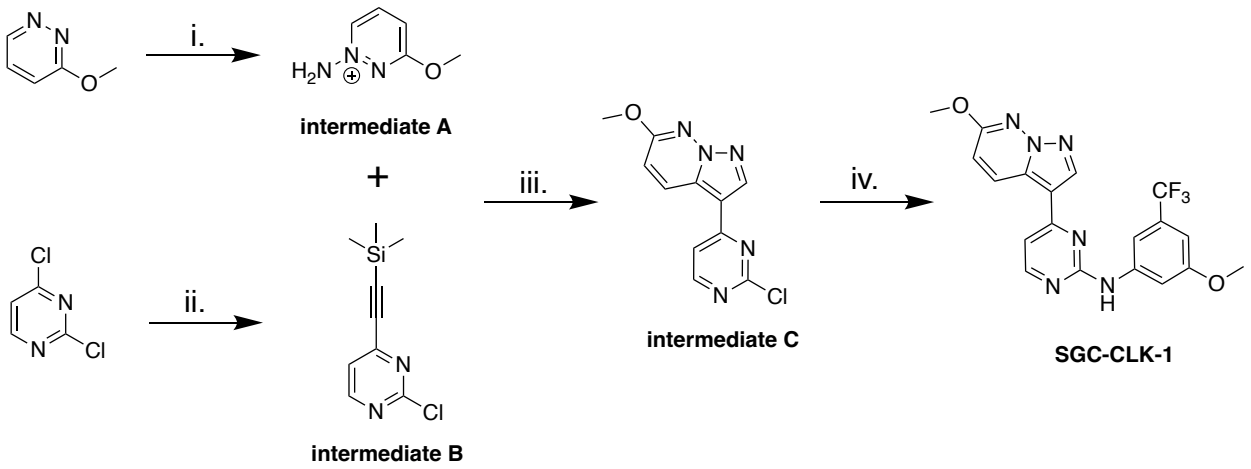
NanoBRET in cell target engagement assays were run as recommended and described by Promega. General NB procedures are provided in the body of the paper. For STK16 an IC_{50}

value of 167 nM could be generated from the curve, however the highest concentration point did not go to the no tracer control. Thus, we describe this as giving only partial inhibition. The other examples depicted here show that the probe SGC-CLK-1 does not bind to HIPK1 or HIPK2 in this cellular assay at these concentrations. The negative control SGC-CLK-1N does not bind to STK16, HIPK1 or HIPK2 in this cellular assay at these concentrations.

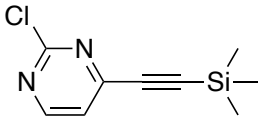
SGC-CLK-1 and SGC-CLK-1N synthesis procedures and compound characterization

General Chemistry Information

All reagents and solvents, unless specifically stated, were used as obtained from their commercial sources without further purification. Air and moisture sensitive reactions were performed under an inert atmosphere using nitrogen in a previously oven-dried or flame-dried reaction flask, and addition of reagents were done using a syringe. All microwave (MW) reactions were carried out in a Biotage Initiator EXP US 400W microwave synthesizer. Thin layer chromatography (TLC) analyses were performed using 200 µm pre-coated sorbtech fluorescent TLC plates and spots were visualized using UV light. High resolution mass spectrometry samples were analyzed with a ThermoFisher Q Exactive HF-X (ThermoFisher, Bremen, Germany) mass spectrometer coupled with a Waters Acquity H-class liquid chromatograph system. Column chromatography was undertaken with a Biotage Isolera One instrument. Nuclear magnetic resonance (NMR) spectrometry was run on a varian Inova 400 MHz or Bruker Avance III 700 MHz spectrometer equipped with a TCI H-C/N-D 5 mm cryoprobe and data was processed using the MestReNova processor. Chemical shifts are reported in ppm with residual solvent peaks referenced as internal standard.



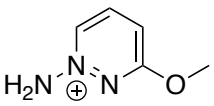
Reagent and conditions: i) Amino hydrogen sulphate, $KHCO_3$, H_2O, 80 C, 14 h; ii) $KHCO_3$, KOH, H_2O, DCM, r.t, 18 h; iii) ETMS, $Pd(dppf)Cl_2 \cdot CH_2Cl_2$, CuI, PPh_3, TEA, THF, 70 C, 15 min; iv) TFA, tert-BuOH, 85 C, 15 h.



2-chloro-4-((trimethylsilyl)ethynyl)pyrimidine (Intermediate B):

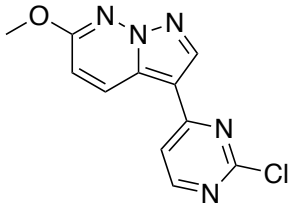
2,4-dichloropyrimidine (1.00 g, 6.7 mmol, 1 eq.) and tetrahydrofuran (30 mL) degassed with nitrogen for 10-12 min, then ethynyltrimethylsilane (0.73 g, 7.4 mmol, 1.1 eq.) and triethylamine (0.74 g, 7.5 mmol, 1.1 eq.) added, degassed for another 5min, then the rest of reagents PdCl2(dppf)-CH2Cl2adduct (0.27 g, 0.34 mmol, 0.05 eq.), copper(I) iodide (0.13 g, 0.67 mmol, 0.10 eq.), triphenylphosphine (0.18 g, 0.67 mmol, 0.10 eq.) were added. Reaction was then refluxed for 15 min. Bulky solid formed. By TLC, all starting material was consumed (Hexanes:EtOAc, 85:15). Hexanes added to precipitated $OPPh_3$, filtered and rinsed with EtOAc. The crude material was concentrated in the rotavap, thick brown liquid formed. Desired product obtained using flash chromatography using EtOAc in hexanes (0% to 20%). Concentrated in rotavapor, dried under vacuum overnight and desired product yielded as brown solid 770 mg (yield 50%), purity>90% by NMR.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.27 (s, 9 H), 7.66 (d, *J*=5.1 Hz, 1 H), 8.80 (d, *J*=5.1 Hz, 1 H).
^{13}C NMR (101 MHz, DMSO-d_6) δ ppm 100.4, 102.1, 122.6, 151.4, 160.1, 161.2.



1-amino-3-methoxypyridazin-1-ium (Intermediate A):

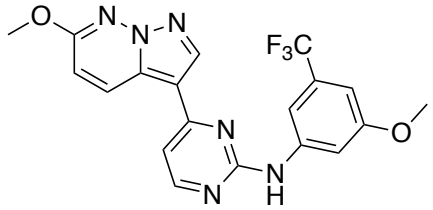
Amino hydrogen sulphate (0.83 g, 7.39 mmol, 3.7 eq.) was dissolved in water (1.6 mL), $KHCO_3$ (0.74 g, 7.39 mmol, 3.7 eq.) in water (1.0 mL), pH 5. Then 3-methoxypyridazine (0.22 g, 2.00 mmol, 1.00 eq.) was added in portion. Reaction was then stirred at 80 °C overnight. This crude is used as is for the next reaction without purification.



3-(2-chloropyrimidin-4-yl)-6-methoxypyrazolo[1,5-b]pyridazine (Intermediate C):

1-amino-3-methoxypyridazin-1-ium (0.20 g, 1.6 mmol, 1.5 eq.) from the previous step (pH = 1) was treated with saturated $KHCO_3$ to bring the pH to 7. 2-chloro-4-((trimethylsilyl)ethynyl)pyrimidine (0.22 g, 1.04 mmol, 1.00 eq.) was dissolved in 1 mL of DCM (1 M), added in one portion over the crude. KOH (0. 320 g, 6.26 mmol, 6 eq.) was dissolved in H_2O (5 mL) 1.0 M and was added in one portion over previous mixture. The reaction mixture was transformed dark read in color after 5-10 min. Reaction mixture was strongly stirred at r.t. for 22 h. The crude mixture was then quenched with water, extracted with DCM, and combined organic layers dried over anhydrous Na_2SO_4. Crude was dry loaded on a 10 g Biotage Sfar 60 um silica cartridge (Hexanes/EtOAc 70:30) and whitish/light pink solid yielded (0.095 g, 35% yield). LCMS [M+1] = 262, purity > 95%.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 4.02 (s, 3 H), 7.27 (d, *J*=9.4 Hz, 1 H), 8.01 (d, *J*=5.5 Hz, 1 H), 8.66 - 8.78 (m, 2 H), 8.83 (s, 1 H).



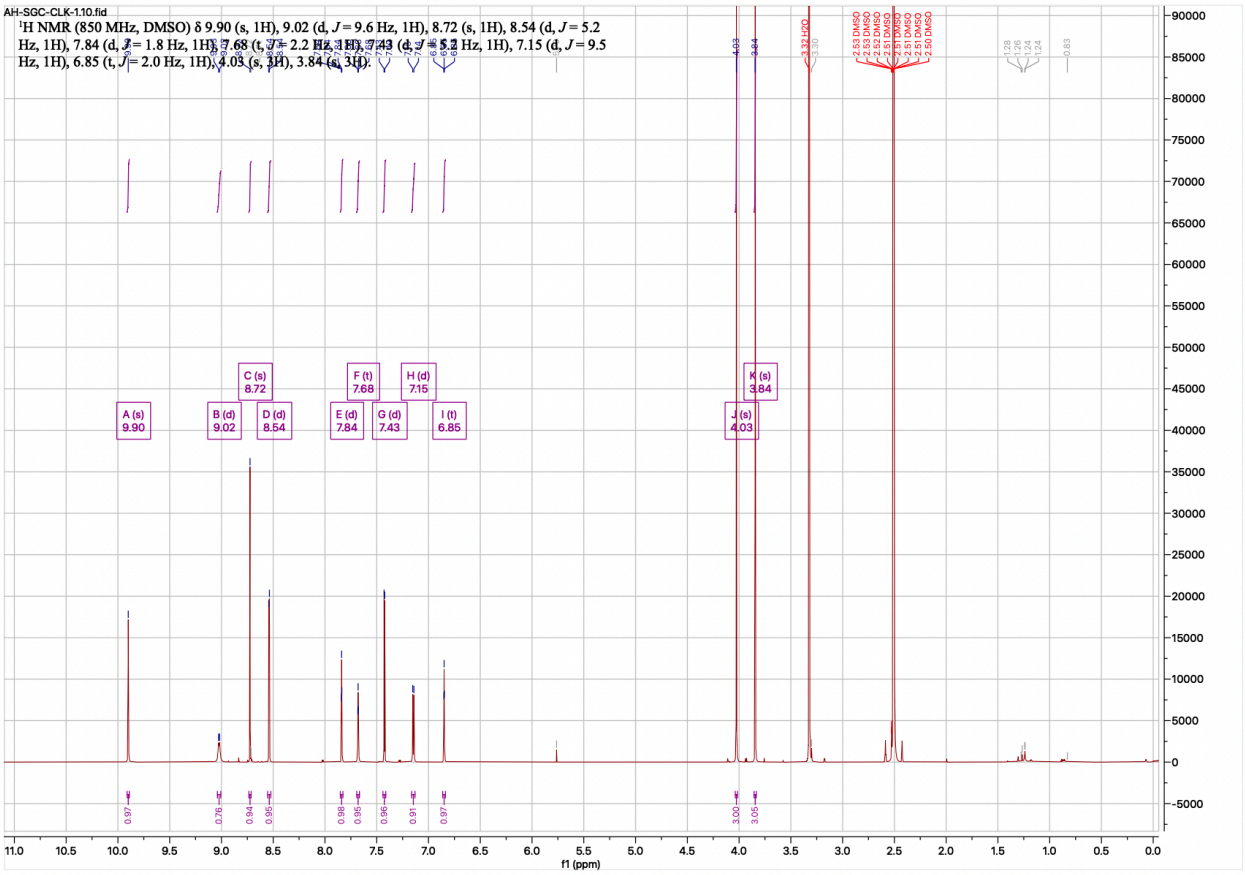
N-(3-methoxy-5-(trifluoromethyl)phenyl)-4-(6-methoxypyrazolo[1,5-b]pyridazin-3-yl)pyrimidin-2-amine (SGC-CLK-1; CAF-170)

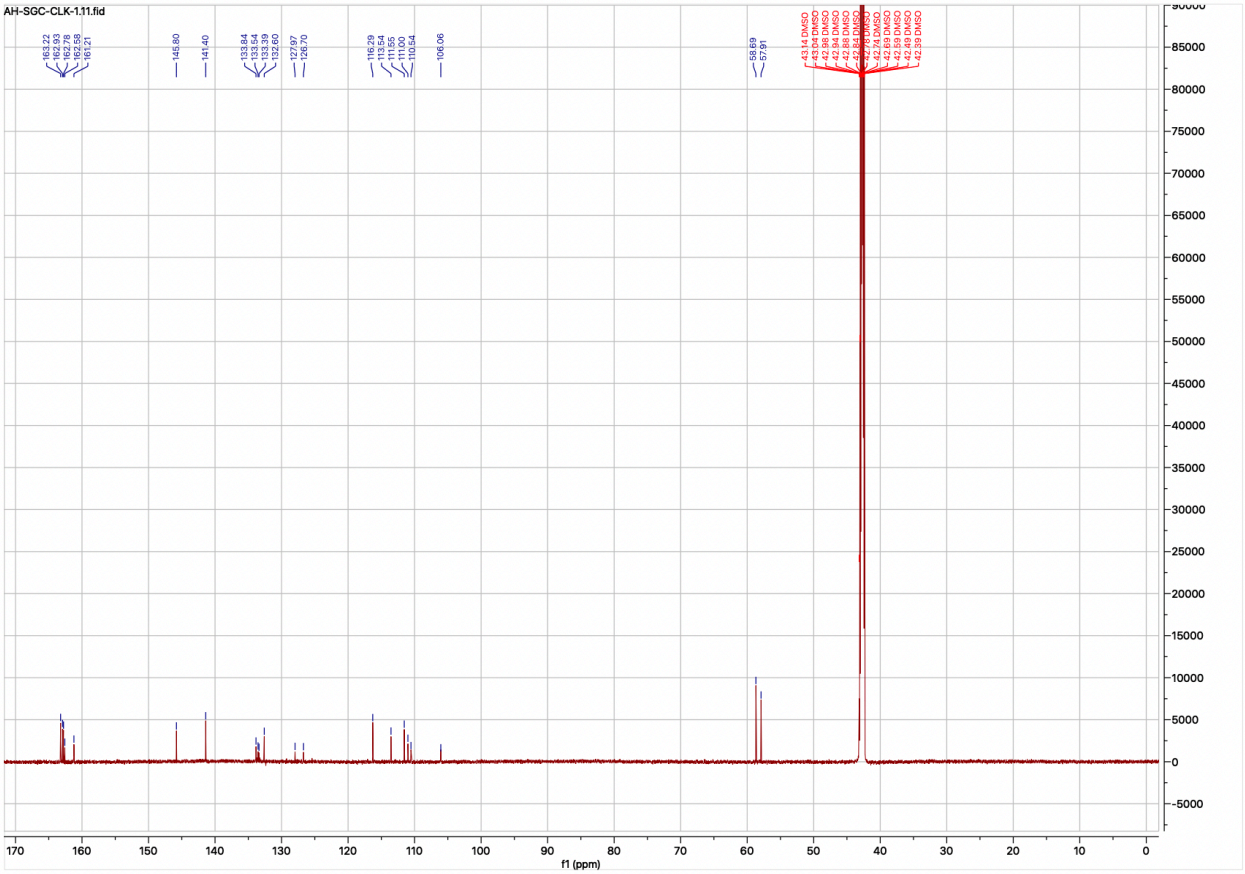
3-(2-chloropyrimidin-4-yl)-6-methoxypyrazolo[1,5-b]pyridazine (0.085 g, 0.32 mmol, 1 eq.), 3-methoxy-5-(trifluoromethyl)aniline (0.075 g, 0.39 mmol, 1.20 eq.), and tert-butanol (4.5 mL) were mixed into a microwave vial, 4 small drops of TFA added, vial sealed, reaction stirred at 85 C for 15 h. Reaction mixture cooled to r.t., quenched with water and $NaHCO_3$, pH adjusted to 7, solid precipitated, more water added, solid filtrated, thoroughly rinsed with water, air dried. Pale pink solid obtained, 110 mg recovered. The product was purified using Biotage Sfar 10g silica

cartridge, solid load. Hexanes/EtOAc gradient from 0% to 50% EtOAc and pale whitish solid yielded (0.057 g, > 95% pure). LCMS [M+1] = 417, 418.

^{1}H NMR (850 MHz, DMSO-d_6) δ 9.90 (s, 1H), 9.02 (d, J = 9.6 Hz, 1H), 8.72 (s, 1H), 8.54 (d, J = 5.2 Hz, 1H), 7.84 (d, J = 1.8 Hz, 1H), 7.68 (t, J = 2.2 Hz, 1H), 7.43 (d, J = 5.2 Hz, 1H), 7.15 (d, J = 9.5 Hz, 1H), 6.85 (t, J = 2.0 Hz, 1H), 4.03 (s, 3H), 3.84 (s, 3H).

^{13}C NMR (214 MHz, DMSO-d_6) δ 163.22, 162.93, 162.78, 162.58, 161.21, 145.80, 141.40, 133.84, 133.54, 133.39, 132.60, 127.97, 126.70, 116.29, 113.54, 111.55, 111.00, 110.54, 106.06, 58.69, 57.91.

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163.22
162.83
162.78
162.58
161.21
145.80
141.40
133.84
133.54
133.39
132.60
127.97
126.70
116.29
113.54
111.35
111.00
110.54
106.06
58.69
57.91
43.14 DMSO
43.04 DMSO
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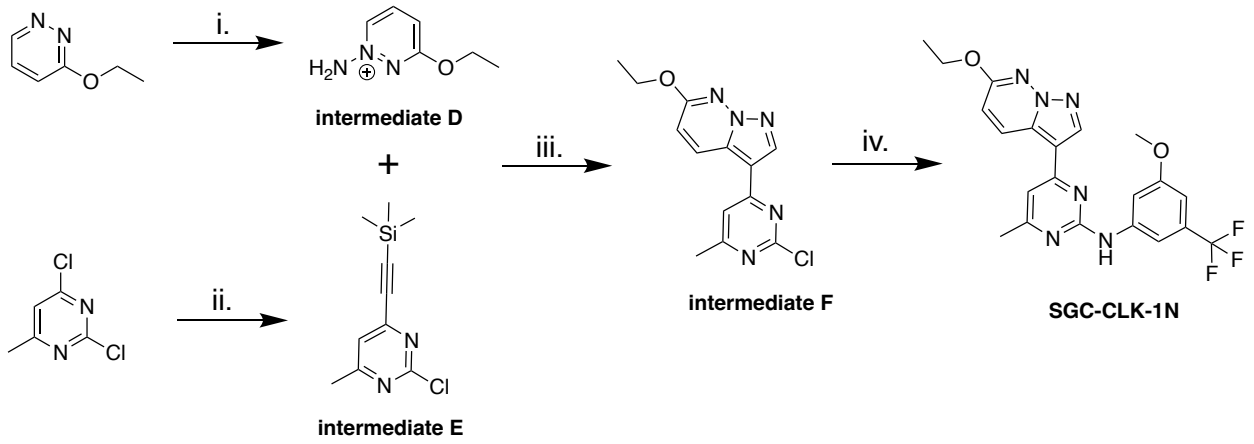
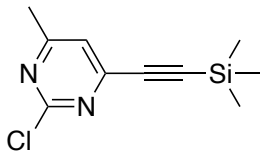
42.84 DMSO
42.74 DMSO
42.69 DMSO
42.59 DMSO
42.49 DMSO
42.39 DMSO
f1 (ppm)

Negative control



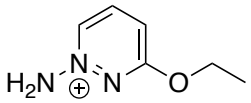
Reagent and conditions: i) Amino hydrogen sulphate, $KHCO_3$, H_2O, 80 C, 14 h; ii) $KHCO_3$, KOH, H_2O, DCM, r.t, 18 h; iii) ETMS, $Pd(dppf)Cl_2 \cdot CH_2Cl_2$, CuI, PPh_3, TEA, THF, 70 C, 15 min; iv) TFA, tert-BuOH, 85 C, 15 h.



2-chloro-4-methyl-6-((trimethylsilyl)ethynyl)pyrimidine (Intermediate E):

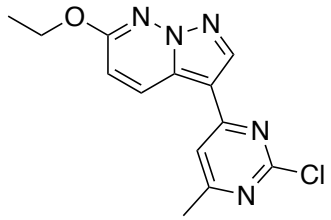
2,4-dichloro-6-methylpyrimidine (1.0 g, 6.10 mmol, 1.00 eq.) and THF (30 mL) degassed with nitrogen for 10-12 min, then ethynyltrimethylsilane (0.66 g, 6.7 mmol, 1.1 eq.) and triethylamine (0.68 g, 6.7 mmol, 1.10 eq., 0.94 mL) added, degassed for another 5 min, then the rest of reagents PdCl2(dppf)-CH_2Cl_2adduct (0.25 g, 0.31 mmol, 0.05 eq.), copper(I) iodide (0.12 g, 0.61 mmol, 0.10 eq.), triphenylphosphine (0.16 g, 0.61 mmol, 0.10 eq.) were added. Reflux kept for 15min. Bulky solid formed. Filtered over celite, rinsed with EtOAc. Concentrated in the rotavapor, tick brown liquid/paste found. The crude product was purified using flash chromatography (0 to 15% EtOAc) 1.025 g (yield 67%), purity around 90%.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.27 (bs, 9 H), 2.46 (s, 3 H), 7.61 (s, 1 H).



1-amino-3-ethoxypyridazin-1-ium (Intermediate D):

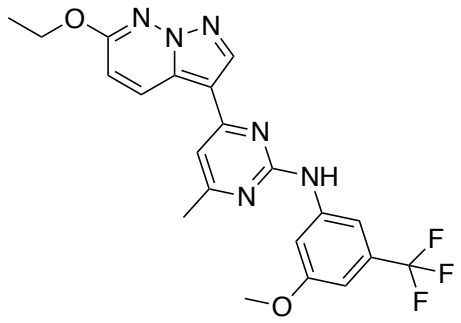
Amino hydrogen sulphate (0.82 g, 7.25 mmol, 4.5 eq.) was dissolved in water (1.6 mL), $KHCO_3$ (0.72 g, 7.25 mmol, 4.5 eq.) in water (1.0 mL), pH 5. Then 3-ethoxypyridazine (0.20 g, 1.61 mmol, 1.00 eq.) was added in portion. Reaction was then stirred at 80 °C overnight. This crude is used as is for the next reaction without purification.



3-(2-chloro-6-methylpyrimidin-4-yl)-6-ethoxypyrazolo[1,5-b]pyridazine (Intermediate F):

3-ethoxy-1l4-pyridazin-1-amine (0.22 g, 1.6 mmol, 2.0 eq.) from the previous step (pH = 1) was treated with saturated $KHCO_3$ to bring the pH to 7. 2-chloro-4-methyl-6-((trimethylsilyl)ethynyl)pyrimidine (0.18 g, 0.88 mmol, 1.00 eq.) was dissolved in 0.8 mL of DCM (1 M), added in one portion over the crude. KOH (0. 27 g, 4.8 mmol, 6 eq.) was dissolved in H_2O (4.8 mL) 0.9-1.0 M and was added in one portion over previous mixture. The reaction mixture was transformed dark read in color after 5-10 min. Reaction mixture was strongly stirred at r.t. for 22 h. The crude mixture was then quenched with water, extracted with DCM, and combined organic layers dried over anhydrous Na_2SO_4. Crude was dry loaded on a 10 g Biotage Sfar 60 um silica cartridge (Hexanes/EtOAc 70:30) and whitish solid yielded (0.116 g, 48% yield). LCMS [M+1] = 290, purity > 95%.

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.41 (t, *J*=7.0 Hz, 3 H), 2.49 (br s, 3 H), 4.41 (q, *J*=7.0 Hz, 2 H), 7.23 (d, *J*=9.4 Hz, 1 H), 7.92 (s, 1 H), 8.69 - 8.77 (m, 2 H).

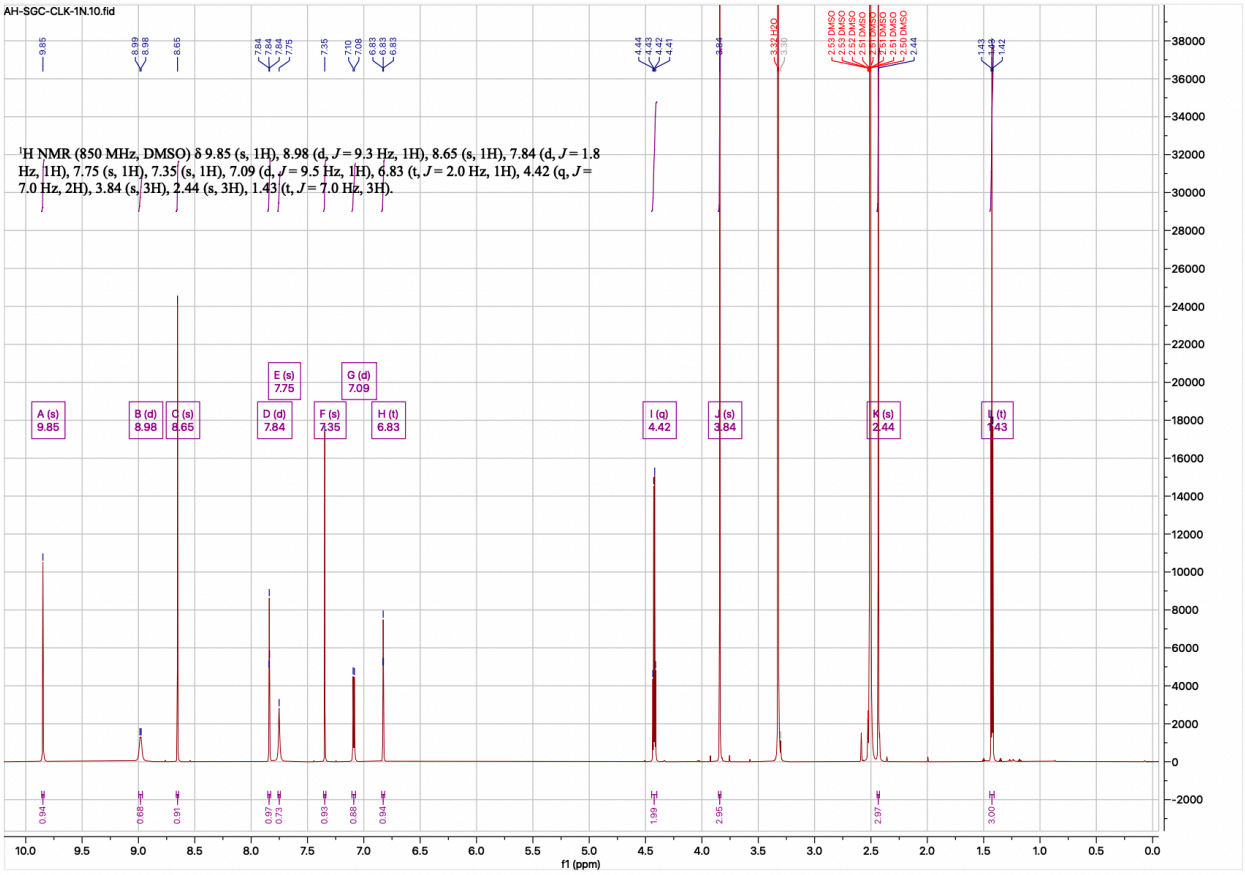


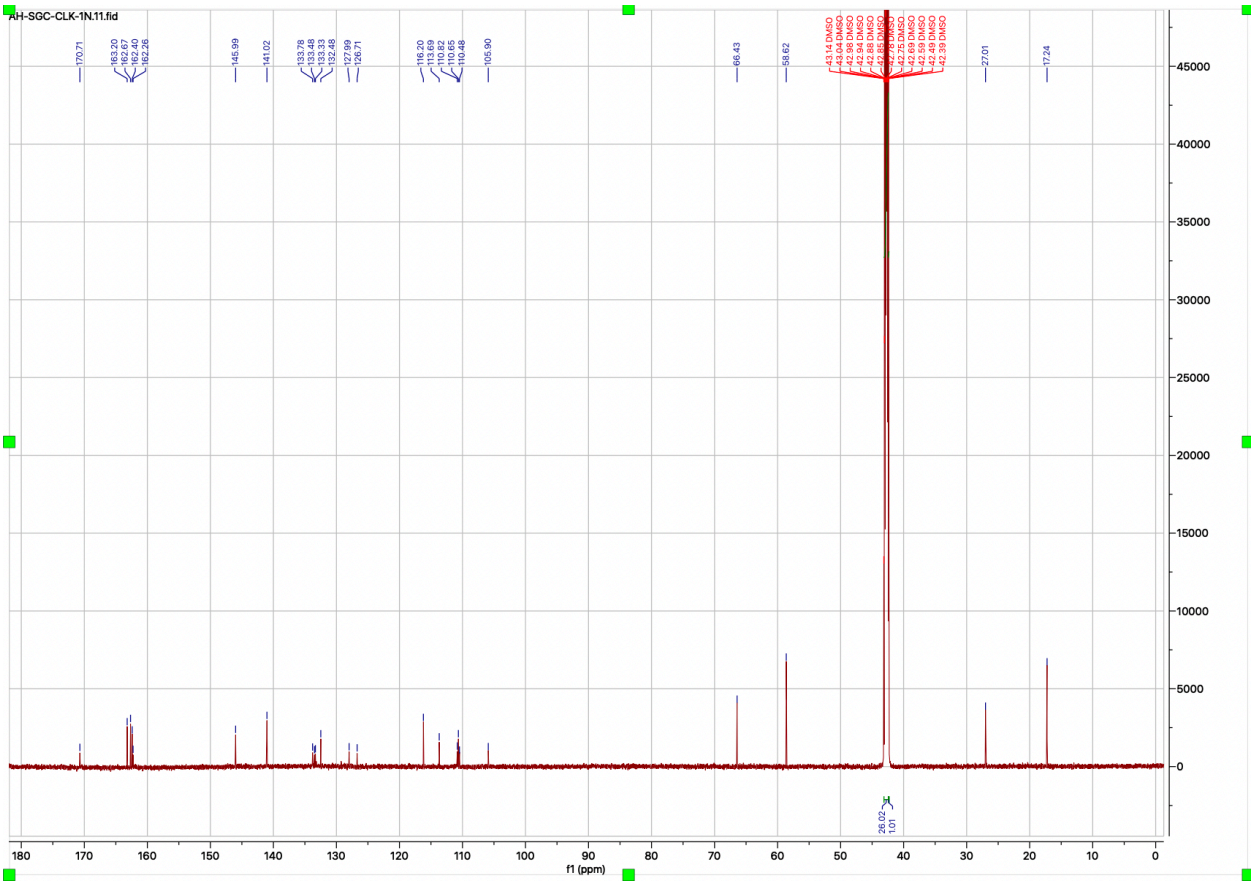
4-(6-ethoxypyrazolo[1,5-*b*]pyridazin-3-yl)-*N*-(3-methoxy-5-(trifluoromethyl)phenyl)-6-methylpyrimidin-2-amine (SGC-CLK-1N, CAF-225):

3-(2-chloro-6-methylpyrimidin-4-yl)-6-ethoxypyrazolo[1,5-b]pyridazine (0.065 g, 0.22 mmol, 1 eq.), 3-methoxy-5-(trifluoromethyl)aniline (0.051 g, 0.27 mmol, 1.20 eq.), and tert-butanol (4.5 mL) were mixed into a microwave vial, 4 small drops of TFA added, vial sealed, reaction stirred at 85 C for 15 h. Reaction mixture cooled to r.t., quenched with water and $NaHCO_3$, pH adjusted to 7, solid precipitated, more water added, solid filtrated, thoroughly rinsed with water, air dried. Pale pink solid obtained, 110 mg recovered. The product was purified using Biotage Sfar 10g silica cartridge, solid load. Hexanes/EtOAc gradient from 0% to 50% EtOAc and pale whitish solid yielded (0.065 g, yield 30%, > 95% pure). LCMS [M+1] = 445, 446.

^{1}H NMR (850 MHz, DMSO-d_6) δ 9.85 (s, 1H), 8.98 (d, J = 9.3 Hz, 1H), 8.65 (s, 1H), 7.84 (d, J = 1.8 Hz, 1H), 7.75 (s, 1H), 7.35 (s, 1H), 7.09 (d, J = 9.5 Hz, 1H), 6.83 (t, J = 2.0 Hz, 1H), 4.42 (q, J = 7.0 Hz, 2H), 3.84 (s, 3H), 2.44 (s, 3H), 1.43 (t, J = 7.0 Hz, 3H).

^{13}C NMR (214 MHz, DMSO-d_6) δ 170.71, 163.20, 162.67, 162.40, 162.26, 145.99, 141.02, 133.78, 133.48, 133.33, 132.48, 127.99, 126.71, 116.20, 113.69, 110.82, 110.65, 110.48, 105.90, 66.43, 58.62, 27.01, 17.24.


AH-SGC-CLK-1N.10.fid
^{1}H NMR (850 MHz, DMSO) δ 9.85 (s, 1H), 8.98 (d, J = 9.3 Hz, 1H), 8.65 (s, 1H), 7.84 (d, J = 1.8 Hz, 1H), 7.75 (s, 1H), 7.35 (s, 1H), 7.09 (d, J = 9.5 Hz, 1H), 6.83 (t, J = 2.0 Hz, 1H), 4.42 (q, J = 7.0 Hz, 2H), 3.84 (s, 3H), 2.44 (s, 3H), 1.43 (t, J = 7.0 Hz, 3H).
A (s) 9.85
B (d) 8.98
C (s) 8.65
D (d) 7.84
E (s) 7.75
F (s) 7.35
G (d) 7.09
H (t) 6.83
I (q) 4.42
J (s) 3.84
K (s) 2.44
L (t) 1.43
f1 (ppm)


AH-SGC-CLK-1N.11.fid
170.71
163.20
162.67
162.40
162.26
145.99
141.02
133.78
133.48
133.33
132.48
127.99
126.71
116.20
113.69
110.82
110.65
110.48
105.90
66.43
58.62
43.14 DMSO
43.04 DMSO
42.98 DMSO
42.93 DMSO
42.88 DMSO
42.83 DMSO
42.78 DMSO
42.73 DMSO
42.69 DMSO
42.59 DMSO
42.49 DMSO
42.39 DMSO
27.01
17.24
26.02
1.01
f1 (ppm)

Original scans for CLK2 blot