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Small molecule binding sites on the Ras:SOS complex can be exploited for inhibition of Ras activation

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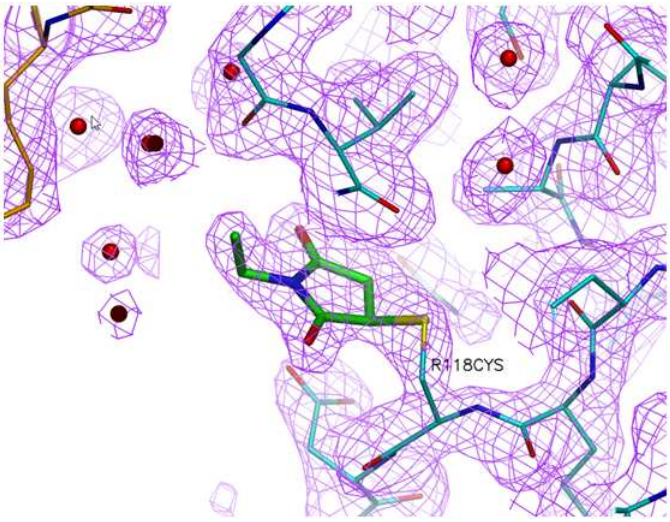
Nuclear magnetic resonance K_D determinations. Samples of 6His-TEV-SH-HRas(1-166) and 6His-TEV-SH-SOS uniformly labeled with $^{2}H,^{15}N$ were produced in *E. coli* using M9 minimal media supplemented with 5 g/L Celtone base powder (^{2}H, 97%, ^{15}N, 98%; Cambridge Isotope Laboratories Inc.), yielding 15 and 150 mg/L of purified protein, respectively. The purification was via NiNTA, TEV cleavage and dialysis, subtractive NiNTA to remove the tag and size exclusion chromatography (SEC) using a Superdex 200 column. After cleavage, both proteins were left with GSH residues preceding the protein sequence. A 16-fold excess of GTPγS was added to the HRas sample to achieve exchange of bound nucleotide, and was then left at room temperature overnight to equilibrate before passing down a PD10 column to remove excess nucleotide. The 2:1 HRas:SOS complex was formed by adding HRas(GTPγS) and SOS from concentrated stocks (~0.8 mM) to obtain 0.5 mL of 0.16 mM HRas and 0.08 mM SOS in 50 mM HEPES (pH 7.4), 50 mM NaCl, 2 mM TCEP, 2 mM $MgSO_4$, 0.1 mM EDTA and 0.02 % NaN_3. NMR spectra were collected at 298 K on a Bruker Avance 600 MHz spectrometer running TopSpin 2.1, equipped with a 5 mm TCI Cryoprobe with Z-axis gradients. The complex was titrated with compound stocks (typically at 200 mM) to give final compound concentrations of 0, 0.2, 0.6, 1.4, 3.4 and 7.4 mM; these were measured using 2D $^{1}H,^{15}N$-TROSY-HSQC[29] experiments recorded with 2048x50 (t_2,t_1) complex points (in Echo-Antiecho mode), and 12019x2068 Hz ($^{1}H,^{15}N$) spectral widths, (85.3 ms x 24.2 ms acquisition times, respectively). The affinity of the compounds was determined via simultaneous nonlinear fitting of fast-exchange chemical shift perturbations (typically 5 amide correlations, employing the absolute value of the linear $^{1}H,^{15}N$ chemical shift vector) against compound concentration using a mass action binding isotherm equation.

Mass spectrometry analysis of covalent adduction to proteins. The HRas:SOS complex was prepared by pre-incubating the purified recombinant HRas with GTPγS (>10 fold molar excess). Both HRas and SOS proteins were transfered individually by gel filtration chromatography into ammonium acetate buffer (20 mM, pH 6.8). Their concentrations were estimated by UV absorbance at 280 nm (NanoDrop, Thermo, Wilmington, DE, USA), before combining the two protein solutions in a stoichiometric ratio of ~2:1 (Ras:SOS) to give final protein concentrations of ~19 μM and ~10 μM respectively. Fragment-like compounds were prepared as solutions at a concentration of 10 mM in either ethanol or acetonitrile. Compound solutions (180 nL) were added to protein solutions (3.25 μL) and

mixed using 10 cycles of aspiration and re-dispensing (Mosquito HTS, TTPLabtech, Hertfordshire, UK). Incubation (2 hours at room temperature) was in sealed 384-well plates. Automated analysis was performed by sampling directly from the incubation plate and infusing using an Advion Nanomate (Advion, Ithaca, NY, USA) mounted on the source of a QToF mass spectrometer (QToF Micro, Waters, Manchester, UK). Data acquisition (~2 minutes) for each sample was initially under non-denaturing ionisation conditions, changing to denaturing conditions approximately halfway through the acquisition. Data were interpreted manually. Those data obtained under denaturing conditions detected quantitative covalent adduction of compound to the individual HRas and SOS subunits. Any observed delta mass increase was calculated (multiplying the Δ m/z by the protein charge state) and validated by comparison with the expected mass increase resulting from the predicted reaction mechanism of the compound. Data obtained under non-denaturing conditions detected covalent adduction, and also modulation of the protein complex quantitative distribution.

Crystallographic system. A dual expression vector encoding HRas 1-166 with an N-terminal TEV protease cleavable hexa-histidine tag and untagged SOS^{cat} 564-1049 was used to direct expression of the HRas:SOS complex in *E.coli*. Following cell lysis, the clarified lysate was batch bound to a nickel NTA resin using a buffer comprised of 40 mM HEPES (pH 8.0), 300 mM NaCl, 20 mM imidazole and 2 mM β-mercaptoethanol, and finally eluted with a step gradient of 500 mM imidazole. The eluted complex was incubated overnight in the presence of TEV protease to remove the affinity tag, whilst dialyzing against a buffer comprised of 40 mM HEPES (pH 8.0), 300 mM NaCl and 2 mM β-mercaptoethanol to reduce the imidazole concentration to 20 mM. Reverse affinity purification was performed to remove the protease and uncleaved material. This was followed with a gel filtration chromatographic step using a Superdex 200 column run in a final buffer of 20 mM TRIS (pH 8.0) and 100 mM NaCl. The peak containing the HRas:SOS complex was pooled, concentrated to 11 mg/mL, and stored at -80 °C. Protein crystallisation was performed using sitting drops at room temperature with 1:1 ratios of protein complex to a mother liquor comprised of 3.8-4.0 M sodium formate and 0.1 M TRIS (pH 8.0). Crystals appeared within a few days and grew to their final size in 1-2 weeks. Cocktail soaking was performed by transferring crystals into a solution containing 4.5 M sodium formate, 5% glycerol and a total cocktail concentration of 50 mM in 20% DMSO, representing 12.5 mM of each fragment. Following one hour exposure, crystals were harvested and frozen for data collection. For single compound soaks, a soaking solution of 4.5 M sodium formate, 0.1 M TRIS (pH 8.0) and 5% glycerol was used with a final

concentration of compound at 10 mM, 10% DMSO. Data were collected at both the European Synchrotron Radiation Facility in Grenoble, France, and Diamond Light Source, Oxford, UK. The search model used during structure solution was *1BKD*. A combination of software from the CCP4 suite[30] and the Global Phasing Consortium[31,32] was used to process the data, and solve and refine the structures. Manual model completion in Coot[33] was used to generate the final models for design. **Supplementary Figure 1** shows an example of electron density that was collected.



Supplementary Figure 1 | Example of electron density data collected. An example of 2Fo-Fc electron density for the covalent addition of small molecules to cysteine 118 of HRas. HRAS and SOS^{cat} are depicted with cyan and orange carbon atoms respectively. The refined final map is contoured at 1σ.

	4URU	4URV	4URW	4URX	4URY	4URZ	4US0	4US1	4US2
Data collection									
Space group	I422	I422	I422	I422	I422	I422	I422	I422	I422
Cell dimensions									
a, *b*, *c* (Å)	149.28, 149.28, 200.52	149.90, 149.90, 200.20	149.41, 149.41, 200.34	149.87, 149.87, 199.70	149.68, 149.68, 200.18	150.21, 150.21, 201.62	149.22, 149.22, 201.04	149.56, 149.56, 199.64	149.71, 149.71, 200.08
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	120 (2.83) *	50.0 (2.58) *	120 (2.76) *	120 (2.48) *	120 (2.47) *	120 (2.24) *	120 (2.17) *	50 (2.65) *	53 (2.48) *
R_{sym} or R_{merge}	0.068 (0.478)	0.091 (0.746)	0.099 (0.503)	0.056 (0.506)	0.076 (0.489)	0.074 (0.49)	0.063 (0.479)	0.121 (0.865)	0.116 (0.759)
$I / \sigma I$	30.7 (5.6)	15.9 (2.5)	12.7 (3.3)	33.4 (5.1)	17.7 (4.6)	17.1 (3.6)	33.2 (6.4)	13.7 (2.3)	11.6 (2.5)
Completeness (%)	100 (100)	97.6 (99)	100 (100)	100 (100)	91.9 (94.7)	90.5 (91.4)	100 (100)	92.9 (94.3)	97.3 (99.0)
Redundancy	12 (12.3)	6.8 (6.9)	6.7 (6.2)	12 (11.3)	7.9 (7.8)	7.2 (7.1)	13.9 (14.1)	7.2 (7.2)	6.9 (6.8)
Refinement									
Resolution (Å)	2.83	2.58	2.76	2.49	2.47	2.25	2.17	2.65	2.48
No. reflections	27366	35039	29346	40039	37338	50173	59613	30842	39224
R_{work} / R_{free}	19.2 / 23.7	19.5 / 23.2	18.2 / 23.0	19.5 / 22.6	19.4 / 22.9	19.1 / 21.2	18.7 / 21.2	19.3 / 23.1	19.2 / 22.8
No. atoms									
Protein	5053	5034	5058	5043	5029	5065	5063	5072	5060
Ligand/ion	17	20	22	26	32	17	9	17	18
Water	71	198	201	195	183	247	493	178	301
B-factors									
Protein	60.4	54.4	46.7	55.4	54.6	54.2	42	51.4	50
Ligand/ion	76	72.9	46.2	60.2	67.4	59.3	56.2	74.9	71
Water	47.5	47.4	39.6	50.3	50.2	52.4	47.8	42.4	47.2
R.m.s. deviations									
Bond lengths (Å)	1.436	1.435	1.417	1.422	1.404	1.441	1.408	1.414	1.414
Bond angles (°)	0.015	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014

Supplementary Table 1 | X-ray crystallographic data collection and refinement statistics. All structures were solved using data from a single crystal.
* Values in parentheses are for highest-resolution shell.

Ras:Raf HTRF assays. Reagents were diluted in 20 mM HEPES, 150 mM NaCl, 5 mM $MgCl_2$, 0.01% Tween-20 (exchange buffer) unless otherwise noted. Results are given in **Supplementary Table 2**. A graphical representation of this assay can be found in **Supplementary Figure 2**.

KRas premix variant. *A-1.* In a low volume 384 well plate (Greiner 784904) 1 µL of compound (1 mM in DMSO, 200 µM final during the pre-incubation step) was incubated with 4 µL biotinylated-KRas-GDP (15 nM in exchange buffer). The reaction was incubated for 2.5 hrs at ambient temperature and then 1 µL of streptavidin europium (Cisbio, 610SAKLB) (750 ng/mL in exchange buffer additionally containing 20 mM DTT), was added. Finally, 4 µL SOS (5 µM in exchange buffer) was added. This mixture was incubated for 4 hours to allow the detection reagents to equilibrate.

B. In a separate 1.5 mL reaction GST-Raf (20 nM) was added to with anti-GST XL665 (4 µg/mL, Cisbio, 61GSTXLA) in exchange buffer additionally containing potassium fluoride (0.1 M) and BSA (0.01% w/v). This mixture was incubated for 4 hours to allow the detection reagents to equilibrate.

C. 3 µL GTPγS (1 mM in exchange buffer) was added to sample *B* and a 10 µL sample of *B*+GTPγS was added to each well of *A-1* to initiate nucleotide exchange. Final assay concentrations were 3 nM biotin-KRas, 1 µM SOS, 1 µM GTPγS, 10 nM GST-Raf, 2 µg/mL anti-GST-XL665 and 37.5 µg/mL streptavidin europium. The plate was read after 60 minutes using an EnVision plate reader (Perkin Elmer) with an excitation wavelength of 320 nm, emission wavelengths of 615 nm and 665 nm, and the data was normalized to the donor emission (615 nm/665 nm x 10^4). An increase in the emission ratio was detected when the europium and XL665 were brought into close proximity by the binding event between activated KRas and Raf. Inhibition values were calculated from control DMSO samples containing no compound (0 % inhibition) or no biotin-KRas (100% inhibition).

KRas:SOS premix variant. *A-2*. In a low volume 384 well plate (Greiner 784904) 1 µL of compound (2 mM in DMSO, 200 µM final during the pre-incubation step) was incubated with 4 µL biotinylated-KRas-GDP (15 nM in exchange buffer) and 5 µL SOS (4 µM in exchange buffer). The reaction was incubated for 2.5 hrs at ambient temperature and then 1 µL of streptavidin europium (Cisbio, 610SAKLB) (750 ng/mL in exchange buffer additionally containing 20 mM DTT), was added. This mixture was incubated for 4 hours to allow the detection reagents to equilibrate. Mixture *B* was prepared and added to *A-2* as previously described in step *C* (*vide supra*).

SOS premix variant. *A-3.* In a low volume 384 well plate (Greiner 784904) 1 µL of compound (1 mM in DMSO, 200µM final during the pre-incubation step) was incubated with 4 µL SOS (5 µM in exchange buffer). The reaction was incubated for 2.5 hrs at ambient temperature and then 1 µL of streptavidin europium (Cisbio,

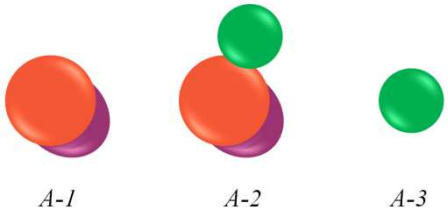
610SAKLB) (750 ng/mL in exchange buffer additionally containing 20 mM DTT), was added. Finally, 4 µL biotinylated-KRas-GDP (15 nM in exchange buffer) was added. This mixture was incubated for 4 hours to allow the detection reagents to equilibrate. Mixture *B* was prepared and added to *A-3* as previously described in step *C* (*vide supra*).

Assay statistics. The assay data varies depending on the pre-incubation conditions (*A-1*, *A-2* or *A-3*) but typical values are in the ranges shown below:

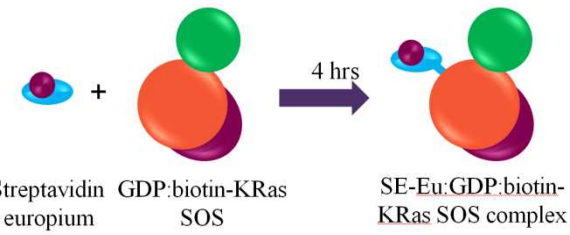
- Signal to noise = 9 – 28
- Signal to background = 2.8 – 4.3
- % CV = 3.6 – 11.5
- Z' = 0.51 – 0.83. Depending on the exact format used Z' values of >0.7 are routinely achieved.
- Range and sensitivity: The theoretical tight-binding limit for compounds targeting KRas is ~1.5 nM, and for SOS binders is 500 nM.

Comment on the high concentration of SOS. The rate of nucleotide exchange will be determined by many factors including the affinity of SOS for the nucleotide bound KRas, the rate of dissociation of GDP, association of GTP, dissociation of SOS and subsequent binding of active KRas to the Raf to generate the HTRF signal. Therefore a pragmatic approach was taken and the SOS was simply titrated into the assay and the initial rate of exchanged was monitored. The concentration selected was approximately half the maximum rate measured. This is analogous to the Km of an enzyme reaction. The 60 minute endpoint selected was on the linear part of the exchange curve. The 1 µM SOS used is not inconsistent with other groups using the MANT assay but this HTRF assay is more sensitive and enables the KRas concentration to be much lower than is required for the MANT assay.

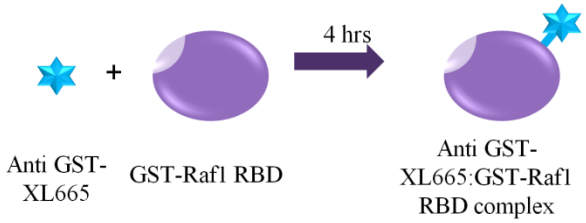
S2a



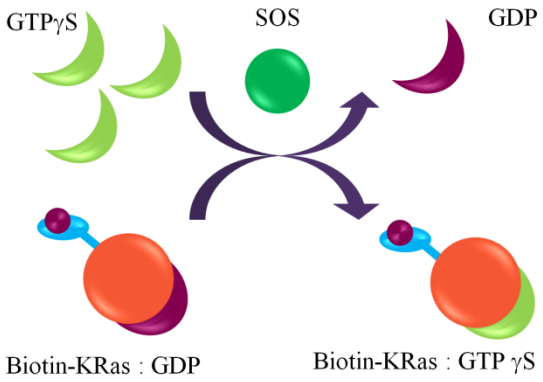
S2b



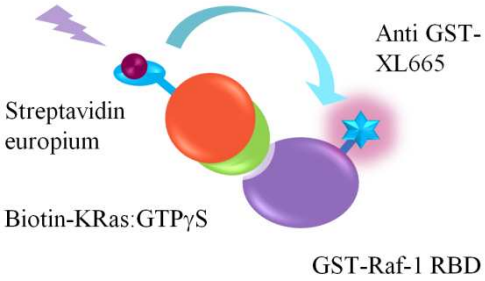
S2c



S2d



S2e



Supplementary Figure 2 | Schematic representation of the HTRF assay. (**a**) *Step A:* Compound pre-incubation. Test compounds were pre-incubated with either GDP:biotinylated KRas alone (*A-1*), GDP:biotinylated KRas + SOS (*A-2*) or SOS alone (*A-3*) for 2.5 hours. Mixtures of GDP, Ras and SOS are assumed to be in equilibrium as Ras:GDP and Ras:SOS but for simplicity are depicted as a single complex in the figure. (**b**) KRas and SOS concentrations in the three sets of pre-incubation conditions were normalized and streptavidin-europium added to each sample. (**c**) *Step B:* GST-Raf and anti-GST were mixed and incubated for 4 hours to allow the detection reagents to equilibrate. (**d**) *Step C:* KRas activation and detection. SOS-mediated nucleotide exchange and detection was initiated by the addition of the GTPγS and simultaneous addition of Raf-1/XL665. (**e**) KRas:GTPγS is detected upon binding to GST-Raf1-RBD which brings the streptavidin-europium and anti-GST-XL665 into close proximity. Compounds which bind to KRas or stabilise the KRas:SOS complex and prevent nucleotide exchange will result in loss of the HTRF signal.

Pre-incubation		**Compounds**					**Controls**	
		10	**11**	**12**	**13**	**14**	**DMSO**	**No KRas**
A-1: KRas:GDP	Measurements	48.430, 35.594, 40.193	14.598, -7.689, -1.225	7.418, -18.160, -1.557	19.557, 12.439	51.6107, 47.1189, 44.8484	1.352, -2.447, 5.955, -4.861	99.485, 101.008, 99.508

	Mean	41.4	1.9	-4.1	16.0	47.9	0.0	100.0
	Stdev	6.5	11.5	13.0	5.0	3.4	4.7	0.9
A-2: KRas:GDP + SOS	Measurements	99.506, 102.565, 101.045	98.255, 96.861, 94.349	100.245, 98.688, 98.279	101.077, 98.247, 99.179	101.611, 100.305, 100.471	2.075, 22.415, 13.311, -18.098, -15.954, -3.749	99.533, 100.288, 100.188
	Mean	101.0	96.5	99.1	99.5	100.8	0.0	100.0
	Stdev	1.5	2.0	1.0	1.4	0.7	16.0	0.4
A-3: SOS	Measurements	2.154, -10.344, -25.342	-21.796, -18.857	-13.096	-36.586, 9.190, 17.839	19.165, 24.963, 44.560	-10.967, 7.401, 5.181, -1.615	100.291, 98.218, 101.495
	Mean	-11.2	-20.3	-13.9	-3.2	29.6	0.0	100.0
	Stdev	13.8	2.1	n/a	29.2	13.3	8.3	1.7

p Values	**Compounds**				
	10	**11**	**12**	**13**	**14**
A-1 : A-2	0.0001	0.0001	0.0002	0.0001	<0.0001
A-2 : A-3	0.0001	<0.0001	-	0.0037	0.0008
A-1 : A-3	0.0039	0.0819	-	0.4467	0.0824

Supplementary Table 2 | Inhibition of KRas:SOS functional activity by irreversible inhibitors 10-14. Inhibition of functional activity was measured following 2.5 hour pre-incubation of **10-14** with either KRas alone, a KRas:SOS mixture, or SOS alone. The mean and standard deviation of measurements are given, except for the SOS/**12** experiment where only one measurement was obtained due to a dispensing failure. p Values are shown comparing each of the three assays with one another.

MANT-dGDP nucleotide exchange assays. Reagents were diluted in 20 mM HEPES, 150 mM NaCl, 5 mM $MgCl_2$, 0.01% Tween-20. Results are given in **Supplementary Table 3**.

MANT-dGDP pre-equilibration. In three separate 400 μL reactions, KRas (1 μM), KRas(G12V) (1 μM) and KRas(G12C) (1 μM) were each incubated with SOS (2 μM) and MANT-dGDP (1.5 μM) (2'-Deoxy- 3'-O-(N'-methylanthraniloyl)guanosine-5'-O-diphosphate, Biolog Life # D084-05). The reactions were incubated for 4 hours to allow the MANT-dGDP to equilibrate with the unlabelled GDP bound to the purified KRas.

KRas inactivation. To monitor the inactivation of KRas, 6 μL of **12** (as a 400 μM solution) was added to a black 384 well plate (Greiner 748900). A portion of the equilibrated KRas:SOS:MANT-dGDP mixture (6 μL) was added to **12** (for a final compound concentration of 200 μM) and the plate was read kinetically (350 nm/450 nm ex/em) using a PHERAstar microplate reader (BMG LABTECH, Germany). Data for each individual well were double referenced to time zero (Time x – time zero fluorescence) and DMSO controls (Compound data – DMSO data at each time point).

	Time (min)	**0**	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**
Ras WT	Mean	0	-1688	-2635	-5120	-5606	-5338	-6460	-5891	-6736
	Std. dev.	0	1016	1230	568	754	935	914	1343	599
Ras (G12V)	Mean	0	-1903	-3874	-5164	-6113	-6877	-7303	-7532	-7519
	Std. dev.	0	358	336	907	726	684	878	959	1041
Ras (G12C)	Mean	0	-3536	-4647	-6489	-7956	-8163	-8371	-9221	-8883
	Std. dev.	0	211	407	1075	724	673	174	839	86
Ras WT (xs GDP)	Mean	0	-1581	-2726	-4640	-5153	-5803	-6367	-6450	-7599
	Std. dev.	0	599	539	725	1059	853	875	1027	1353

	Time (min)	**45**	**50**	**55**	**60**	**65**	**70**	**75**	**80**	**85**
Ras WT	Mean	-6669	-6607	-7348	-6796	-8022	-7327	-8486	-9225	-8592
	Std. dev.	1505	1609	1685	679	1458	1762	1745	1972	1493
Ras (G12V)	Mean	-7800	-7502	-7806	-7477	-8715	-8711	-9593	-9742	-9994
	Std. dev.	1027	767	847	1037	1039	636	863	1079	929
Ras (G12C)	Mean	-9274	-9570	-9753	-9019	-9999	-10285	-10921	-11361	-11571
	Std. dev.	531	269	467	720	406	1176	519	231	949
Ras WT (xs GDP)	Mean	-7551	-7358	-7942	-7911	-8479	-8304	-8126	-8629	-8220
	Std. dev.	1615	1520	1820	1718	2009	1910	2119	2235	2148

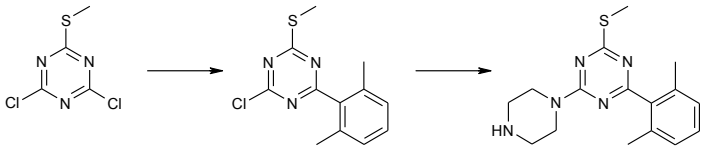
	Time (min)	**80**	**85**	**90**	**95**	**100**	**105**	**110**	**115**	**120**
Ras WT	Mean	-9225	-8592	-9627	-10048	-10261	-10423	-10318	-10639	-10646
	Std. dev.	1972	1493	1879	1666	1810	2054	2128	2198	1535
Ras (G12V)	Mean	-9742	-9994	-9985	-10652	-10683	-10779	-11188	-11272	-11113
	Std. dev.	1079	929	868	503	1576	759	847	1094	904

Ras (G12C)	Mean	-11361	-11571	-12449	-11510	-12975	-12291	-13238	-13202	-13152
	Std. dev.	231	949	1141	542	1155	742	990	956	650
Ras WT (xs GDP)	Mean	-8629	-8220	-8419	-8431	-8258	-8522	-8452	-8410	-8650
	Std. dev.	2235	2148	2571	2688	2530	2535	2583	2783	2815

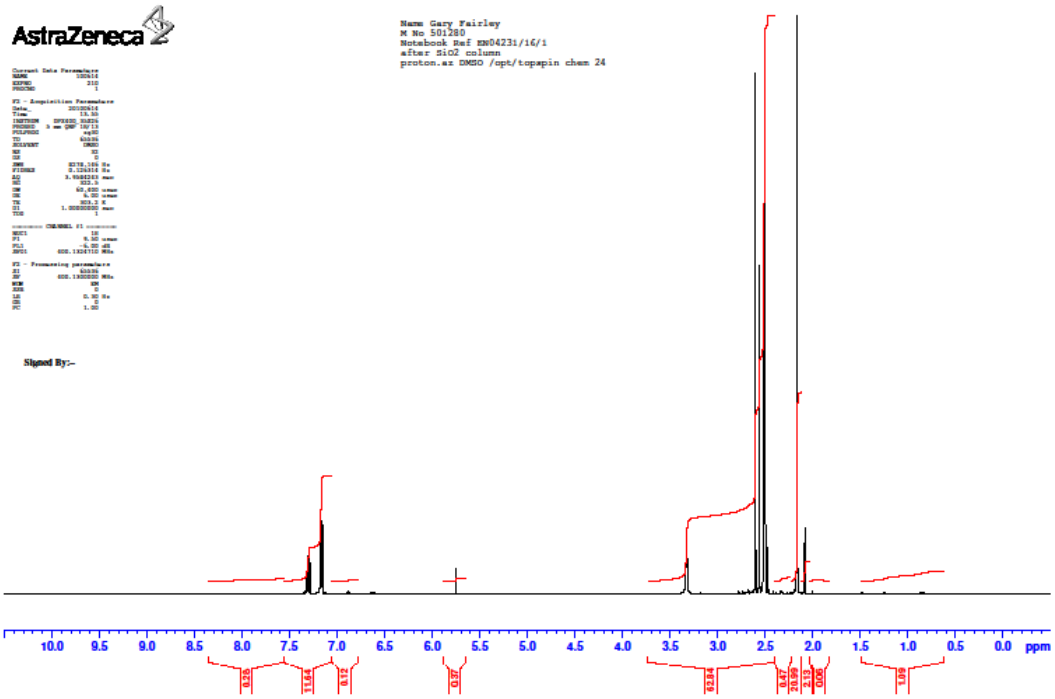
Supplementary Table 3 | Inhibition of SOS-mediated MANT-labelled nucleotide exchange by 12, with wild type Ras, and the Ras mutants G12C and G12V. The fourth experiment contained no **12**, and instead an excess of unlabelled GDP was added. This competes with the MANT-dGDP, acts as a control for the expected loss of fluorescence upon MANT-dGDP displacement and represents the rate of exchange of GDP under these experimental conditions.

Compound synthesis and characterisation

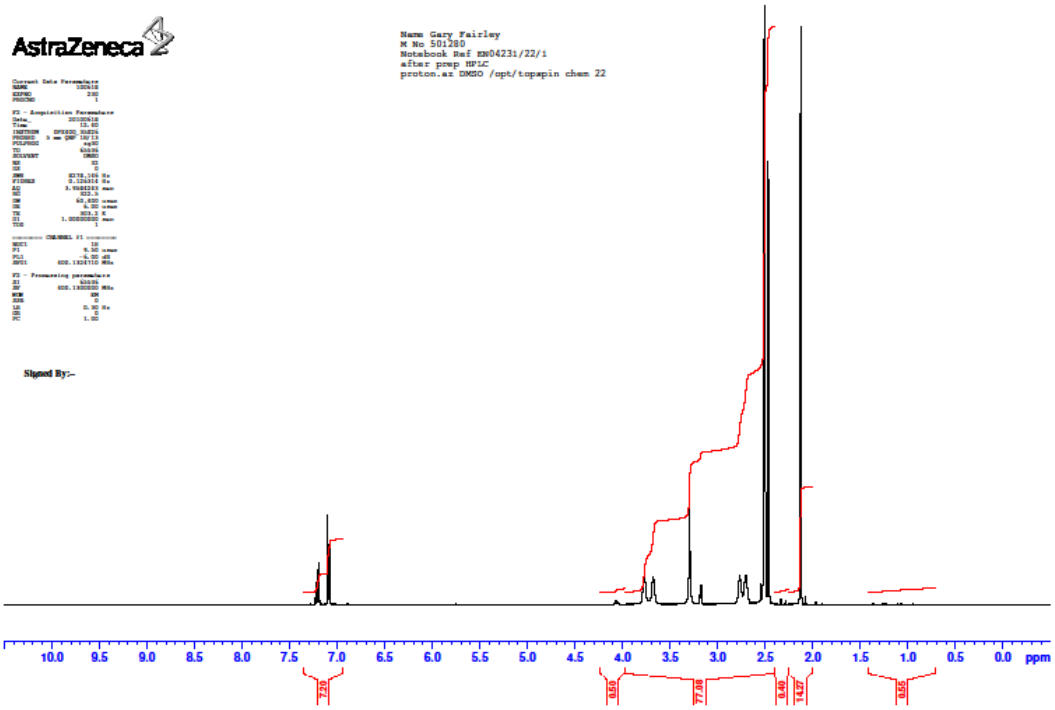
1, **2**, **4a**, **4b**, **8**, **9** & **10** are commercially available. Synthesis of **7** is described in reference (5). Synthesis and characterisation of novel compounds **3**, **5**, **6**, **11**, **12**, **13** and **14** is described below.

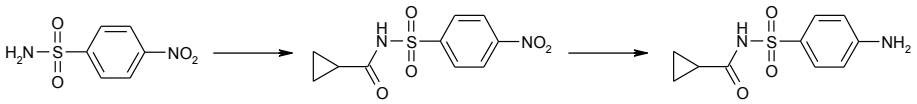


2-(2,6-Dimethylphenyl)-4-methylsulfanyl-6-piperazin-1-yl-1,3,5-triazine, 3. A 1 M solution of (2,6-dimethylphenyl)-magnesium bromide in tetrahydrofuran (5.1 mL, 5.1 mM) was added dropwise to 2,4-dichloro-6-(methylthio)-1,3,5-triazine (1.00 g, 5.1 mM) in tetrahydrofuran (2.5 mL) at 25 °C over a period of 1 minute under nitrogen. The resulting mixture was stirred at 67 °C for 18 hours. The reaction mixture was allowed to cool to room temperature and then quenched with saturated ammonium chloride (5 mL), diluted with ethyl acetate and water, shaken, and the organic layer was collected. The organic layer was then washed with saturated brine, dried over magnesium sulfate, filtered and evaporated to dryness to give crude product (1.37 g). The crude product was purified by flash silica chromatography (80 g column, Grace), with an elution gradient of 5 to 40% dichloromethane in isohexane. Pure fractions were evaporated to dryness to afford 2-chloro-4-(2,6-dimethylphenyl)-6-(methylthio)-1,3,5-triazine (0.788 g, 58.1 %) as a yellow oil which solidified on standing under high vacuum overnight. MS (*m/z*): $[MH]^+$ = 266.4; ^{1}H NMR (400 MHz, DMSO-d_6, 30 °C): δ 7.34 - 7.28 (m, 1H), 7.17 (d, *J* = 7.8 Hz, 2H), 2.60 (s, 3H), 2.16 (s, 6H).

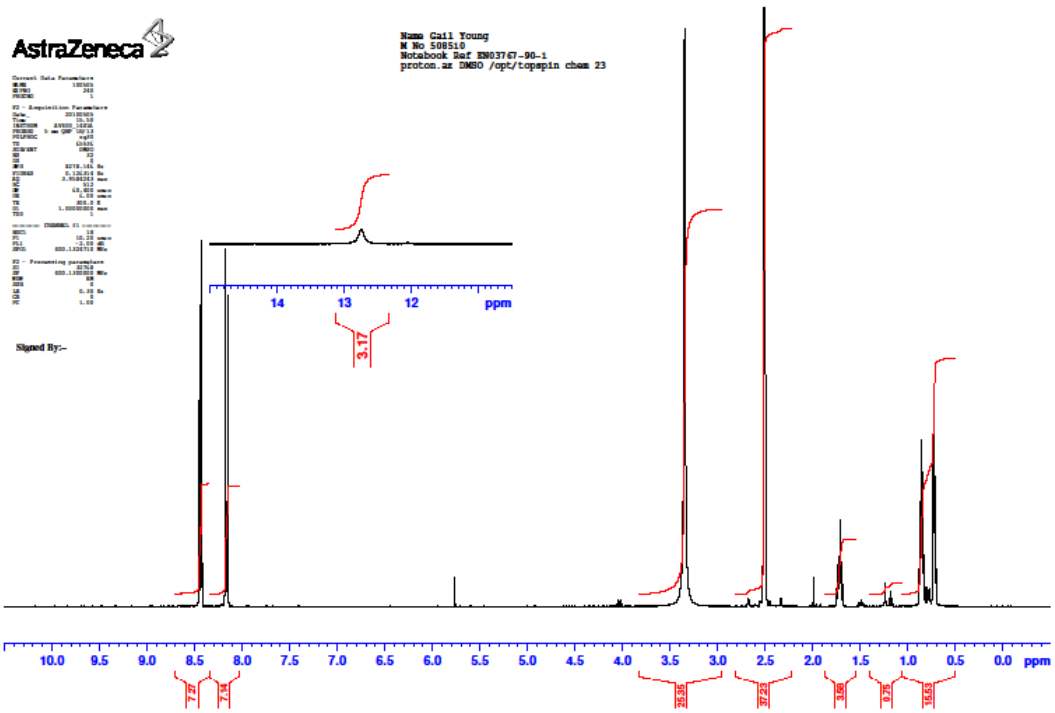


t-Butyl piperazine-1-carboxylate (0.126 g, 0.68 mM) was added in one portion to 2-chloro-4-(2,6-dimethylphenyl)-6-(methylthio)-1,3,5-triazine (0.150 g, 0.56 mM) and *N*-ethyl-*N*-isopropylpropan-2-amine (0.187 mL, 1.13 mM) in 2-pentanol (2.6 mL) at 25 °C. The resulting mixture was stirred at 100 °C for 35 minutes and allowed to cool to room temperature. The reaction mixture was evaporated to dryness, dissolved in dichloromethane (2 mL) and trifluoroacetic acid (2 mL) was added dropwise to the stirred reaction mixture at 25 °C. The reaction mixture was evaporated to dryness and the crude product was purified by ion exchange chromatography, using a 10 g SCX-2 column. The desired product was eluted from the column using a solution of ammonia in methanol (7 M) and product fractions were evaporated to dryness to afford crude product (0.163 g). The crude product was purified by preparative HPLC (Waters XBridge Prep C18 OBD column, 5µ silica, 19 mm diameter, 100 mm length), using decreasingly polar mixtures of water (containing 1% ammonia) and acetonitrile as eluents. Fractions containing the desired compound were evaporated to dryness to afford **3** (0.094 g, 52.9 %) as a colourless gum. HRMS (*m/z*): $[MH]^+$ calcd. for $C_{16}H_{21}N_5S$: 316.15904; found 316.15912; ^{1}H NMR (400 MHz, DMSO-d_6, 21 °C): δ 7.21 (dd, J = 8.0, 7.1 Hz, 1H), 7.09 (d, J = 7.5 Hz, 2H), 3.82 - 3.63 (m, 4H), 2.81 - 2.67 (m, 4H), 2.47 (s, 3H), 2.13 (s, 6H); ^{13}C NMR (126 MHz, DMSO-d_6, 30 °C): δ 180.44, 172.59, 162.11, 137.73, 134.56, 128.17, 127.39, 45.35, 44.32, 44.20, 19.44, 12.57.

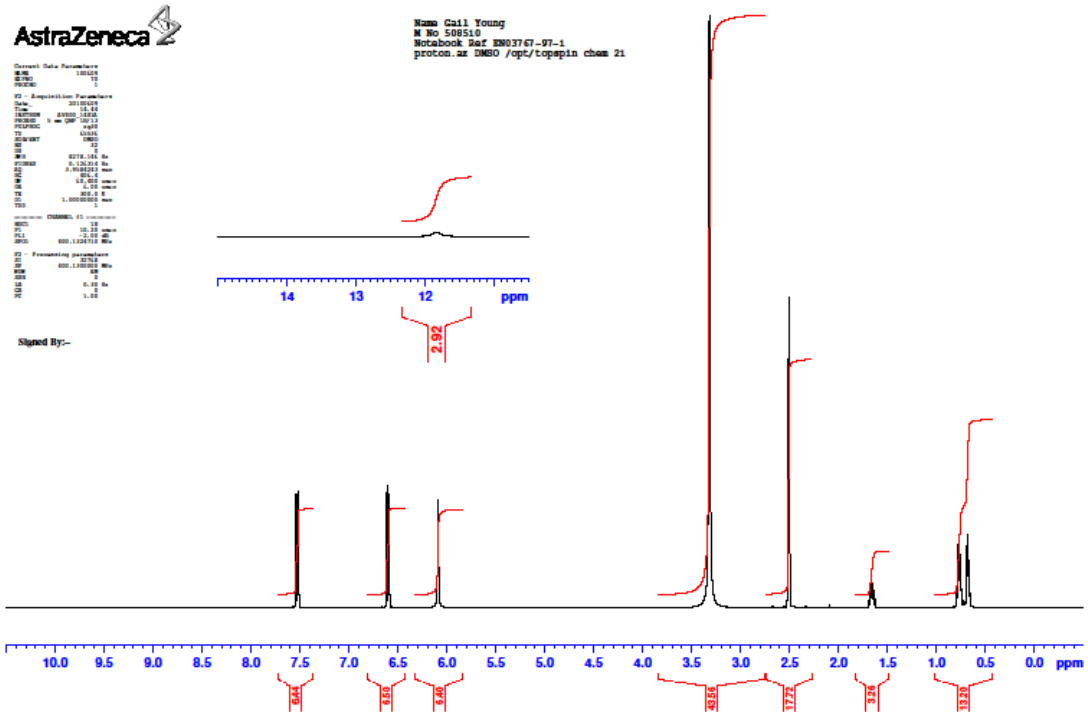


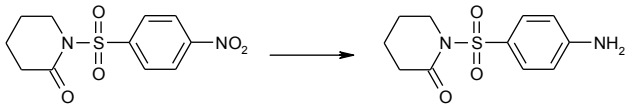


***N*-(4-Aminophenyl)sulfonylcyclopropanecarboxamide, 5.** Cyclopropanecarbonyl chloride (0.114 mL, 1.26 mM) was added to 4-nitrobenzenesulfonamide (0.254 g, 1.26 mM), triethylamine (0.437 mL, 3.14 mM) and *N*,*N*-dimethylpyridin-4-amine (0.015 g, 0.13 mM) in THF (6 mL) at room temperature under nitrogen. The resulting mixture was stirred at ambient temperature for 1 hour. The reaction mixture was diluted with water (50 mL) and washed with 2 N HCl (50 mL). The aqueous layer was removed and the organics further washed with water (50 mL), then brine (50 mL), and finally dried over magnesium sulfate and concentrated under reduced pressure onto silica. The crude product was purified by flash silica chromatography, with an elution gradient of 0 to 5 % methanol in dichloromethane. Pure fractions were evaporated to dryness to afford *N*-(4-nitrophenylsulfonyl)cyclopropanecarboxamide (0.350 g, 100 %) as a cream solid. MS (*m/z*): $[MH]^-$ = 269.35; ^{1}H NMR (400 MHz, DMSO-d_6, 21 °C): δ 12.10 - 12.00 (m, 1H), 8.43 (d, *J* = 8.9 Hz, 2H), 8.16 (d, *J* = 8.9 Hz, 2H), 1.75 - 1.66 (m, 1H), 0.85 (dq, *J* = 7.1, 3.7, 3.7, 3.4 Hz, 2H), 0.74 - 0.68 (m, 2H).

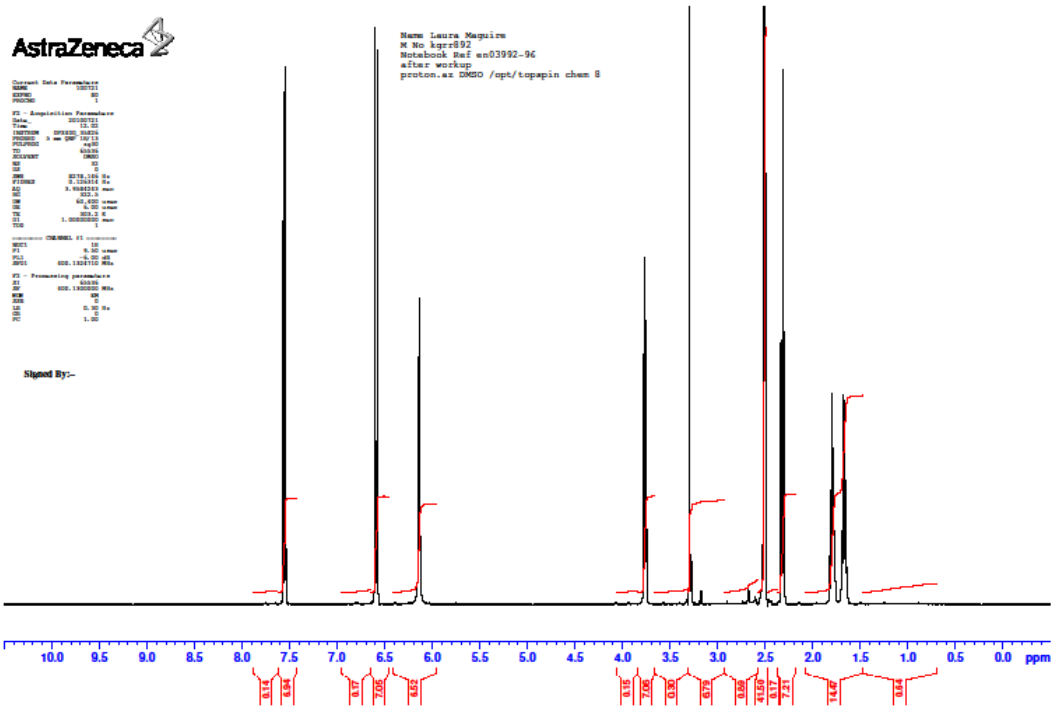


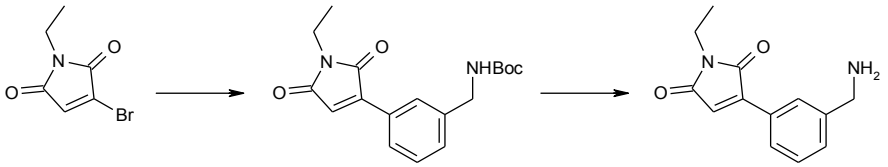
N-(4-Nitrophenylsulfonyl)cyclopropanecarboxamide (0.290 g, 1.08 mM) was added to a reactor tube and dissolved in ethyl acetate (5 mL). This tube was evacuated and purged with nitrogen three times. Palladium 10% on carbon (0.017 mg, 0.02 mM) was then added in a single portion and the flask evacuated and purged with nitrogen a further three times and with hydrogen twice. The suspension was then stirred under an atmosphere of hydrogen at 25 °C for 18 hours. The reaction mixture was filtered through celite then the crude product was purified by preparative HPLC (Waters SunFire column, 5µ silica, 19 mm diameter, 100 mm length), using decreasingly polar mixtures of water (containing 0.1% formic acid) and acetonitrile as eluents. Fractions containing the desired compound were evaporated to dryness to afford **5** (0.202 g, 78 %) as a white solid. HRMS (*m/z*): $[MH]^+$ calcd. for $C_{10}H_{12}N_2O_3S$: 241.06414; found 241.06416; ^{1}H NMR (400 MHz, DMSO-d_6, 21 °C): δ 11.83 (s, 1H), 7.52 (d, J = 8.7 Hz, 2H), 6.59 (d, J = 8.7 Hz, 2H), 6.08 (s, 2H), 1.73 - 1.58 (m, 1H), 0.80 - 0.72 (m, 2H), 0.72 - 0.64 (m, 2H); ^{13}C NMR (126 MHz, DMSO-d_6, 30 °C): δ 171.65, 153.50, 129.66, 123.84, 112.21, 13.59, 8.17.





1-(4-Aminophenyl)sulfonylpiperidin-2-one, 6. A mixture of palladium 10% on carbon (0.344 g, 0.32 mM) and 1-(4-nitrophenylsulfonyl)piperidin-2-one (0.918 g, 3.23 mM) were stirred in a solution of dimethylformamide (30 mL) under an atmosphere of hydrogen at 25 °C for 24 hours. The reaction mixture was filtered, concentrated and diluted with ethyl acetate (75 mL), and washed sequentially with water (2 x 75 mL) and saturated brine (75 mL). The organic layer was dried over magnesium sulfate, filtered and evaporated to afford desired product, which was triturated with acetonitrile, filtered and evaporated to dryness to give **6** (0.610 g, 74 %). HRMS (*m/z*): $[MH]^+$ calcd. for $C_{11}H_{14}N_2O_3S$: 255.07979; found 255.07985; ^{1}H NMR (400 MHz, DMSO-d_6, 21 °C): δ 7.57 - 7.51 (m, 2H), 6.62 - 6.55 (m, 2H), 6.13 (s, 2H), 3.79 - 3.72 (m, 2H), 2.31 (t, J = 6.7 Hz, 2H), 1.79 (ddd, J = 9.6, 7.3, 4.7 Hz, 2H), 1.71 - 1.61 (m, 2H); ^{13}C NMR (126 MHz, DMSO-d_6, 30 °C): δ 169.61, 153.72, 130.31, 130.20, 123.28, 112.09, 112.00, 46.30, 33.66, 22.72, 19.71.

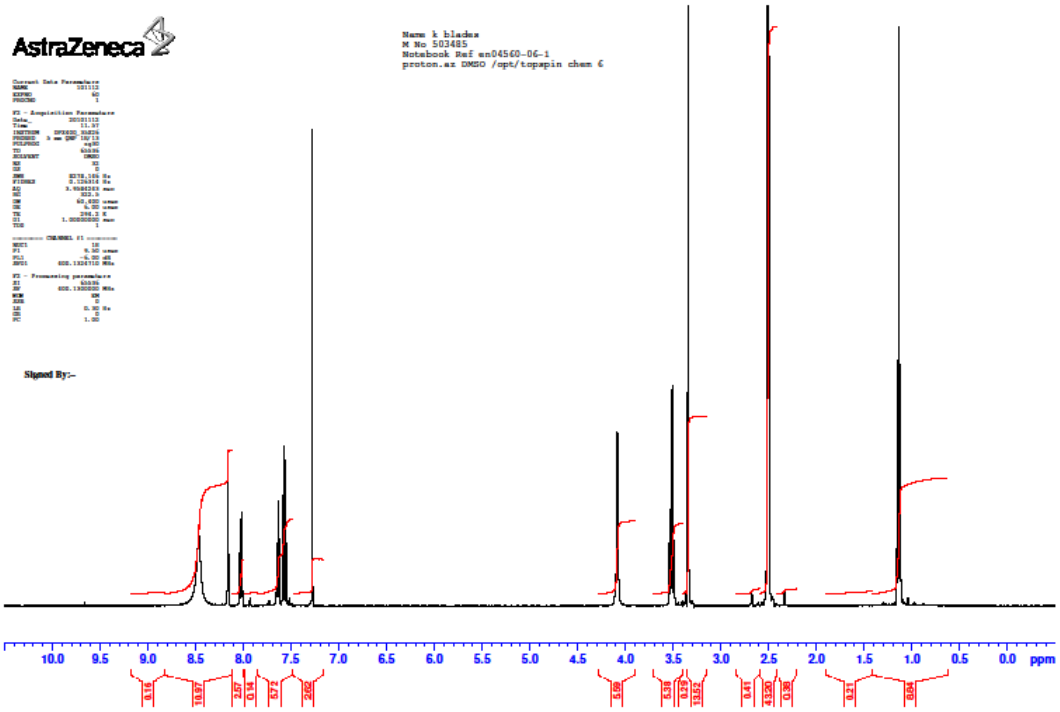


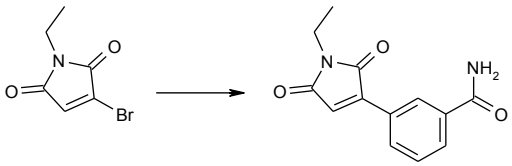


3-[3-(Aminomethyl)phenyl]-1-ethyl-pyrrole-2,5-dione, 11. 3-Bromo-1-ethyl-1H-pyrrole-2,5-dione (0.250 g, 1.23 mM) was added to 4-((*tert*-butoxycarbonylamino)-methyl)phenylboronic acid (0.308 g, 1.23 mM), $PdCl_2$(dppf) (0.067 g, 0.09 mM) and cesium fluoride (0.372 g, 2.45 mM) in dioxane (10 mL) at 20 °C. The reaction was degassed with nitrogen for 10 minutes and then heated at 70 °C for 1 hour. The reaction mixture was filtered through celite and the filtrate was concentrated *in vacuo*. The crude product was purified by flash silica chromatography with an elution gradient of 50 to 100% diethyl ether in heptane. Pure fractions were evaporated to dryness to afford *tert*-butyl 4-(1-ethyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)benzylcarbamate (0.305 g, 75 %) as a yellow gum.

tert-Butyl 3-(1-ethyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)benzylcarbamate (0.320 g, 0.97 mM) was added to 6.0 N HCl in propan-2-ol (10 mL) and the reaction was stirred for 1 hour. Diethyl ether (10 mL) was then added to the reaction and the mixture was stirred for 30 minutes to afford a solid which was collected by filtration and dried under vacuum to give **11** (0.102 g, 39.5 %) as a white solid. HRMS (*m/z*): $[MH]^+$ calcd. for $C_{13}H_{14}N_2O_2$: 231.11280 ; found 231.11285; ^{1}H NMR (400 MHz, DMSO-d_6, 21 °C): δ 8.47 (s, 2H), 8.16 (s, 1H), 8.03 (dt, *J* =

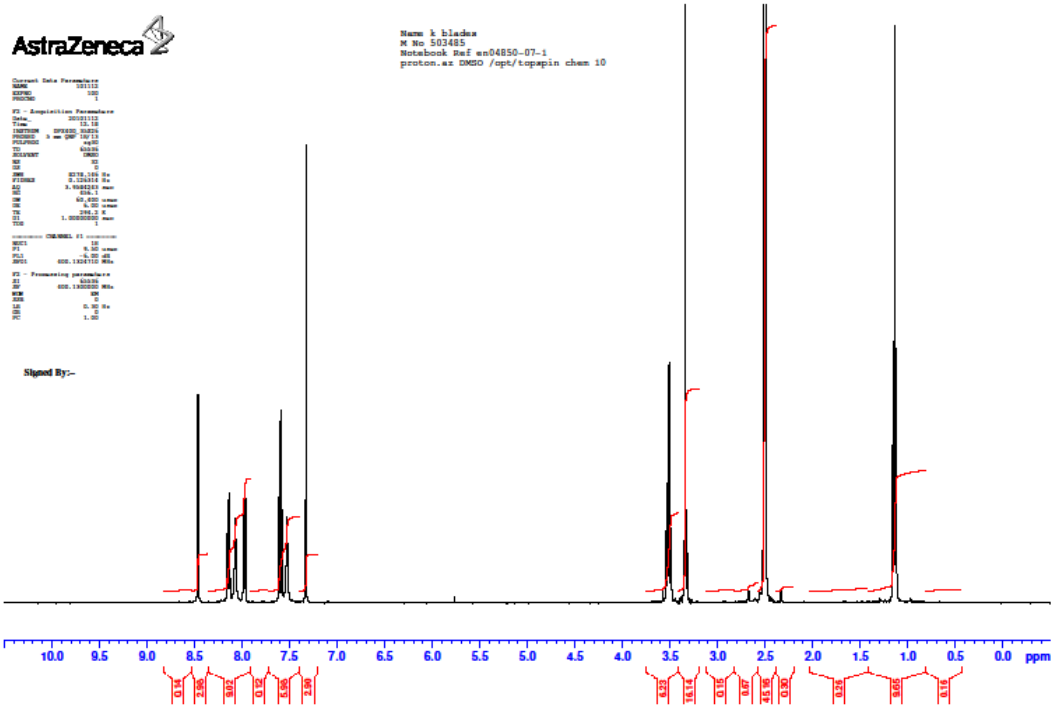
7.7, 1.3 Hz, 1H), 7.64 (d, J = 7.8 Hz, 1H), 7.56 (dd, J = 7.7, 7.7 Hz, 1H), 7.28 (s, 1H), 4.08 (s, 2H), 3.51 (q, J = 7.2 Hz, 2H), 1.14 (t, J = 7.2 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6, 30 °C): δ 170.2, 169.9, 142.2, 134.8, 131.2, 129.0, 129.1, 128.4, 125.5, 42.0, 32.3, 13.6.

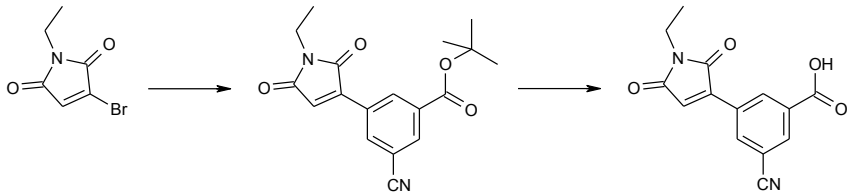




3-(1-Ethyl-2,5-dioxo-pyrrol-3-yl)benzamide, 12. 3-Bromo-1-ethyl-1H-pyrrole-2,5-dione (0.250 g, 1.23 mM) was added to 3-carbamoylphenylboronic acid (0.22 g, 1.35 mM), cesium fluoride (0.372 g, 2.45 mM) and $PdCl_2$(dppf) (0.067 g, 0.09 mM) in dioxane (10 mL)/water (1.0 mL). The reaction was heated to 70 °C for 1 hour, then quenched with water (50 mL). The resulting mixture was extracted with dichloromethane (2 x 50 mL) then the organic layers were dried over magnesium sulfate, filtered and evaporated to afford a brown solid. The crude solid was triturated with dichloromethane to give a solid which was collected by filtration and dried under vacuum to give **12** (0.150 g, 50 %) as a white solid. HRMS (*m/z*): $[MH]^+$ calcd. for $C_{13}H_{12}N_2O_3$: 231.11280; found 231.11285; ^{1}H NMR (400 MHz, DMSO-d_6, 21 °C): δ 8.46 (s, 1H), 8.14 (d, J = 7.8 Hz, 1H), 8.07 (s, 1H), 7.98 (d, J = 7.8 Hz, 1H), 7.60 (t, J = 7.8 Hz, 1H), 7.53 (s, 1H), 7.33 (s, 1H), 3.51 (q, J = 7.2 Hz, 2H), 1.14 (t, J =

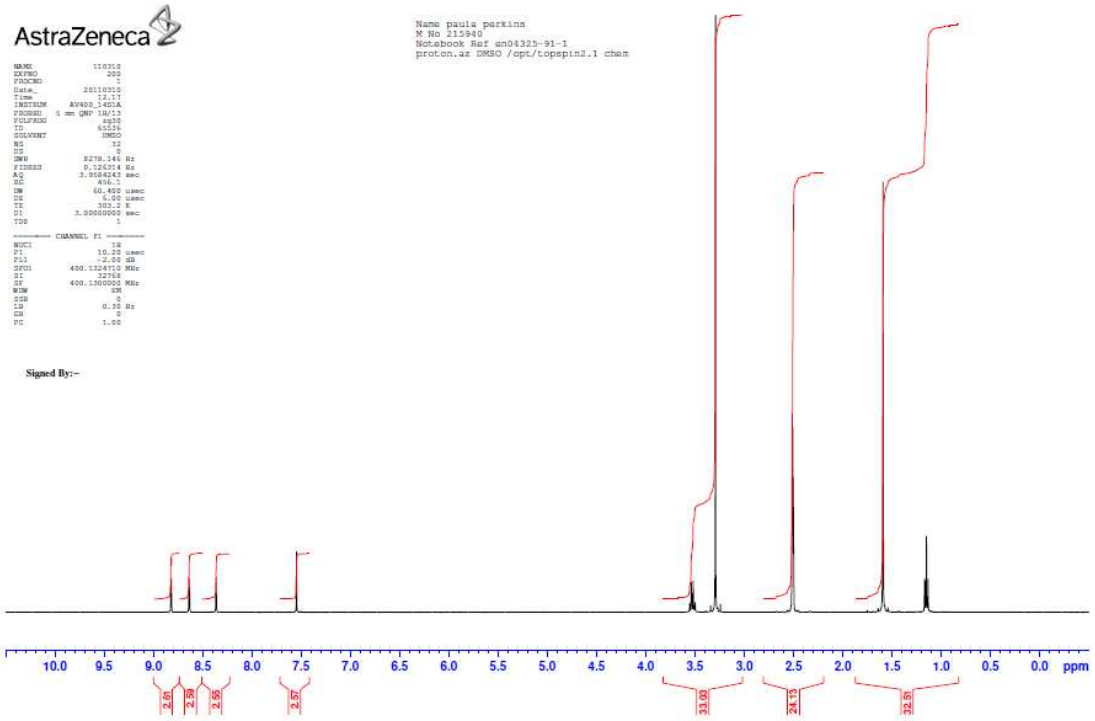
7.2 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6, 30 °C): δ 170.2, 170.0, 167.20, 142.2, 134.9, 131.0, 129.5, 128.9, 128.8, 127.7, 125.8, 32.3, 13.6.



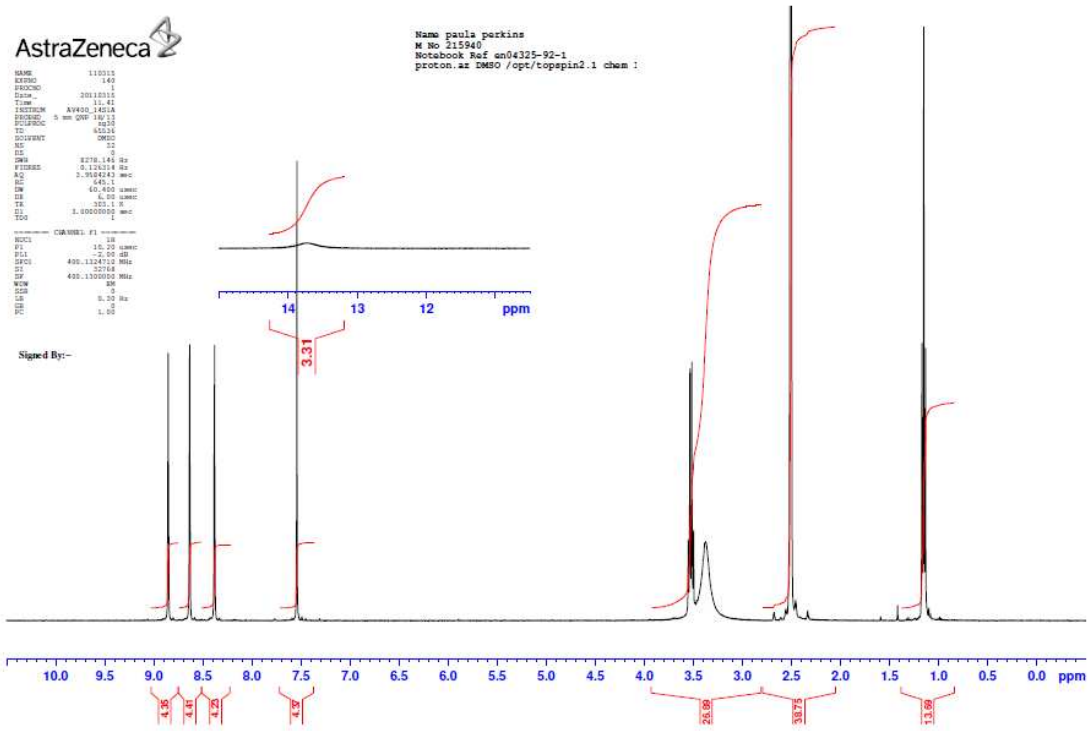


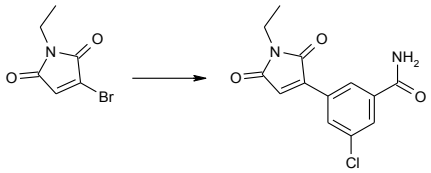
3-Cyano-5-(1-ethyl-2,5-dioxo-pyrrol-3-yl)benzoic acid, 13. *tert*-Butyl 3-cyano-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzoate (0.538 g, 1.23 mM) was added to $PdCl_2$(dppf) (0.067 g, 0.09 mM), 3-bromo-1-ethyl-1H-pyrrole-2,5-dione (0.250 g, 1.23 mM) and cesium fluoride (0.372 g, 2.45 mM) in dioxane (15 mL) at 20 °C. The reaction was degassed with nitrogen for 10 minutes and was then heated at 70 °C for 2 hours. Water (1 mL) was added and the mixture was heated a further 2 hours at 70 °C. The reaction mixture was filtered through celite. The filtrate was concentrated and the residue redissolved in dichloromethane. The crude product was purified by flash silica chromatography with an elution gradient of 0 to 30% ethyl acetate in heptane. Pure fractions were evaporated to dryness to afford *tert*-butyl 3-cyano-5-(1-ethyl-2,5-dioxo-2,5-dihydro-*1H*-pyrrol-3-yl)benzoate (0.250 g, 63 %) as a pale yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6, 21 °C): δ 8.82 (t, J = 1.6 Hz,

1H), 8.64 (t, *J* = 1.6 Hz, 1H), 8.36 (t, *J* = 1.5 Hz, 1H), 7.55 (s, 1H), 3.53 (q, *J* = 7.2 Hz, 2H), 1.59 (s, 9H), 1.15 (t, *J* = 7.2 Hz, 3H).

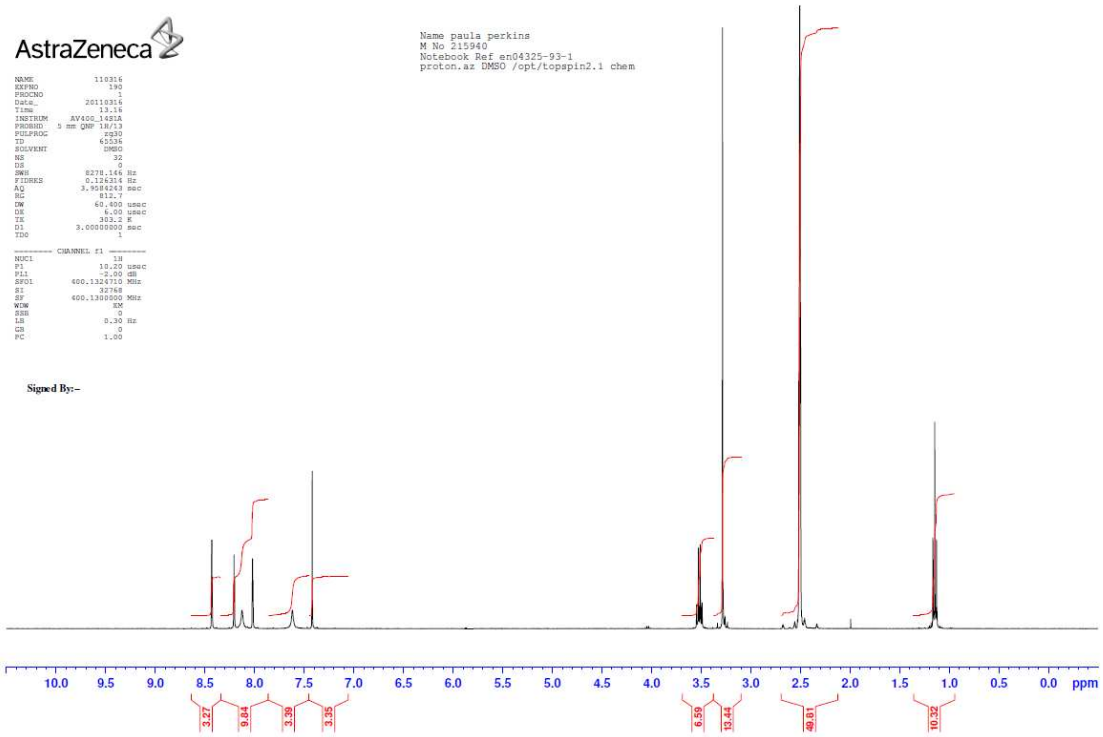


Trifluoroacetic acid (3.00 mL) was added dropwise to a solution of *tert*-butyl 3-cyano-5-(1-ethyl-2,5-dioxo-2,5-dihydro-*1H*-pyrrol-3-yl)benzoate (0.235 g, 0.72 mM) in dichloromethane (6 mL) cooled to 0 °C, then allowed to warm to ambient temperature and stirred for 1.5 hours. The reaction mixture was evaporated to dryness to afford product as an off-white solid, then triturated with diethyl ether and filtered. The resulting solid was dried under vacuum at 40 °C overnight to afford **13** (0.112 g, 58 %) as a white solid. HRMS (*m/z*): $[MH]^-$ calcd. for $C_{14}H_{10}N_2O_4$: 269.05568; found 269.05701; ^{1}H NMR (400 MHz, DMSO-d_6, 21 °C): δ 13.72 (s, 1H), 8.85 (t, *J* = 1.5 Hz, 1H), 8.64 (t, *J* = 1.5 Hz, 1H), 8.38 (t, *J* = 1.4 Hz, 1H), 7.54 (s, 1H), 3.53 (q, *J* = 7.2 Hz, 2H), 1.15 (t, 3H); ^{13}C NMR (101 MHz, DMSO-d_6, 30 °C): δ 169.78, 169.56, 165.16, 139.74, 135.70, 133.98, 133.06, 132.64, 130.57, 127.87, 117.45, 112.64, 32.43, 13.52.





3-Chloro-5-(1-ethyl-2,5-dioxo-pyrrol-3-yl)benzamide, 14. 3-Carbamoyl-5-chlorophenylboronic acid (0.244 g, 1.23 mM) was added to $PdCl_2$(dppf) (0.067 g, 0.09 mM), 3-bromo-1-ethyl-1H-pyrrole-2,5-dione (0.250 g, 1.23 mM) and cesium fluoride (0.370 g, 2.45 mM) in dioxane (15 mL) and water (1 mL) at 20 °C. The reaction was degassed with nitrogen for 10 minutes and was then heated at 70 °C for 2 hours. The reaction mixture was filtered through celite. The filtrate was concentrated and the residue redissolved in dichloromethane. The crude product was purified by flash silica chromatography with an elution gradient of 40 to 100% ethyl acetate in heptane. Pure fractions were evaporated to dryness and then triturated with diethyl ether to afford **14** (0.104 g, 31 %) as a pale yellow solid. HRMS (*m/z*): $[MH]^-$ calcd. for $C_{13}H_{11}N_2O_3Cl$: 277.03870; found 277.03854; ^{1}H NMR (400 MHz, DMSO-d_6, 21 °C): δ 8.43 (t, *J* = 1.5 Hz, 1H), 8.21 (t, *J* = 1.7 Hz, 1H), 8.13 (s, 1H), 8.01 - 8.03 (m, 1H), 7.62 (s, 1H), 7.42 (s, 1H), 3.52 (q, *J* = 7.2 Hz, 2H), 1.15 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6, 30 °C): δ 169.87, 169.68, 165.78, 140.63, 136.68, 133.52, 130.82, 130.32, 128.90, 127.04, 126.39, 32.38, 13.56.



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