G-quadruplex-binding benzo[a]phenoxazines down-regulate c-KIT expression in human gastric carcinoma cells

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Inventory of Supporting Information

Results Figures and Tables:

Figures S1 & S2 illustrate the features of both the *c-KIT* promoter target plasmid (*pGLCKIT*) and the control plasmid (*pRL-TK*) used in the dual luciferase-screening assay

Figure S3 illustrates the *c-KIT* minimal promoter showing the two G-quadruplexes c-kit1 and c-kit2.

Figure S4 indicates how the background levels of *renilla* luciferase are used to determine that tested molecules are not cytotoxic or affecting promoters through non-specific (duplex DNA binding) mechanisms in the reporter assay.

Figure S5 illustrates example Surface Plasmon Resonance (SPR) sensorgrams.

Table S1 illustrates qPCR data for *c-KIT* expression in HGC-27 cells after treatment with small molecules (see also Figure 3).

Table S2 shows the K_d for small molecule binding to G-quadruplexes and double stranded DNA as calculated using SPR (see also Figure 4).

Materials and Methods:

All supplementary methods used, including the procedures and scheme for synthesising **1** and **2**, are presented.

Figures S5-7 show ^{1}H-NMR for small molecules **1**, **2** and **4**.

Results Figures and Tables

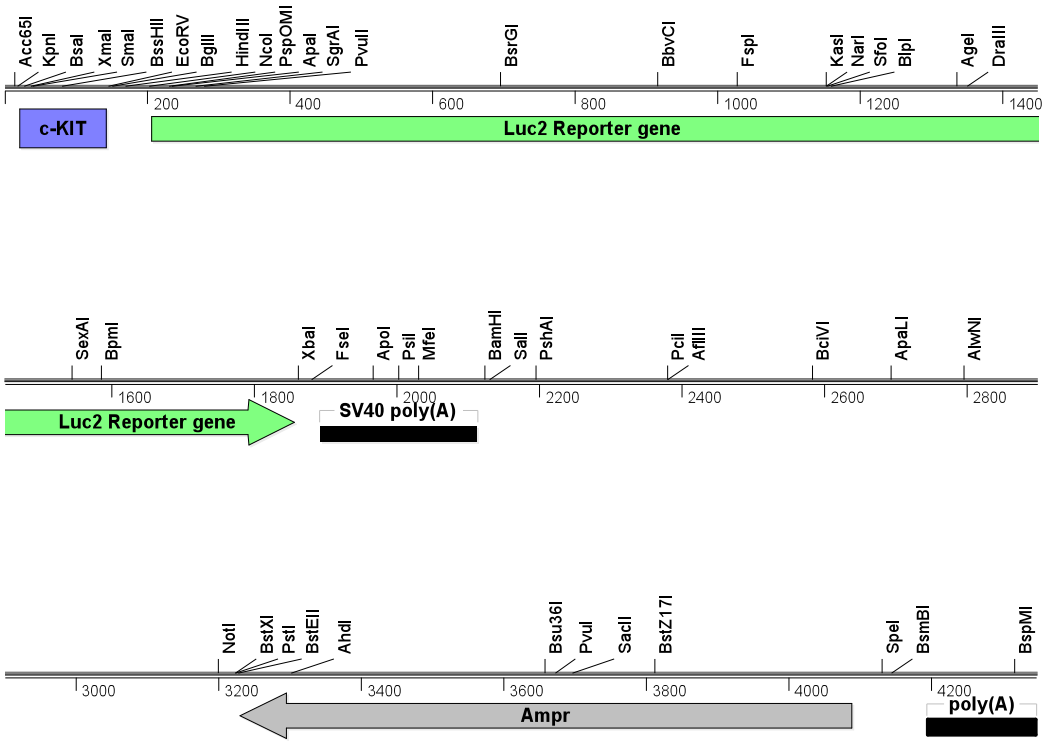


Figure S1. Illustrates the linearised feature map for the *pGL4.10* plasmid construct containing the *c-KIT* minimal promoter driving firefly luciferase transcription (*pGLCKIT*; 4347 bp). All unique restriction sites are shown, where the *c-KIT* promoter region (denoted by 'c-KIT') extends from the Acc65I to EcoRV restriction sites in the multiple cloning region (MCS; bp 1-175). Poly(A) denotes polyadenylation signal/transcriptional pause sites and Ampr is the ampicillin resistance gene.

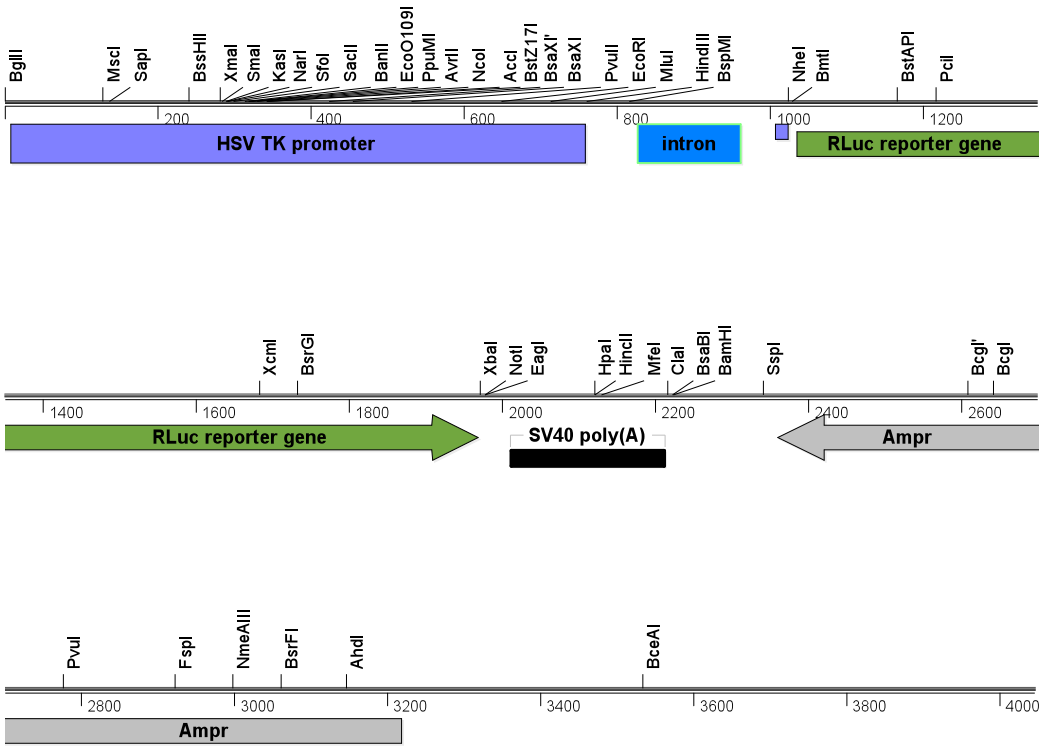


Figure S2. Illustrates the linear feature map for the control plasmid containing the Herpes Simplex Virus thymidine kinase promoter driving *renilla* luciferase gene transcription (*pRL-TK*; 4045 bp). All unique restriction sites are also shown. Poly(A) denotes polyadenylation signal/transcriptional pause sites and Ampr is the ampicillin resistance gene.

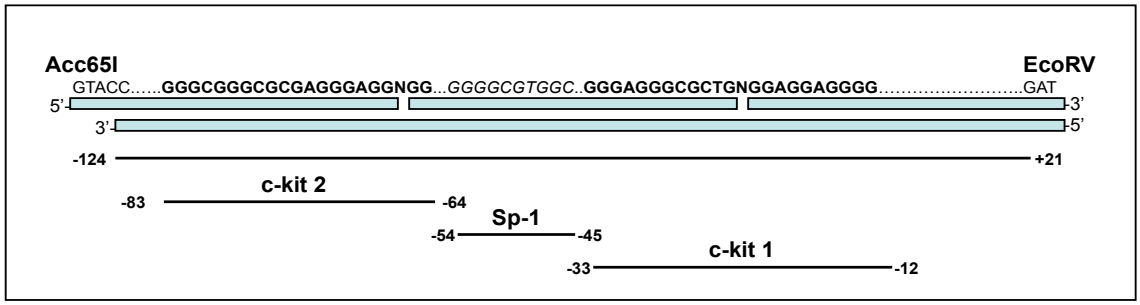


Figure S3. Map of the minimal *c-KIT* promoter insert used in the firefly reporter construct. N denotes 'nick' site on top strand. c-kit2, Sp-1/AP-2 and c-kit1 sequences are indicated. Based on KIT (ENSG00000157404), Ensemble Build v57, Mar 2010.

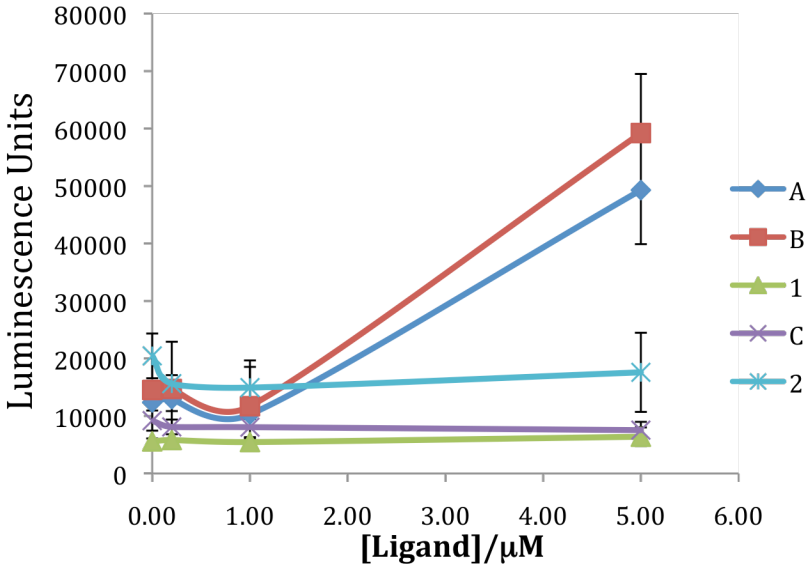


Figure S4. *Renilla* luminescence levels for the 5 most active molecules identified in the reporter assay. From these results Ligand A and Ligand B were not analysed further as the changes in their *renilla* levels indicate probable cytotoxicity. Ligand C was not analysed further as it did not provide reproducible results in the dual luciferase screen.

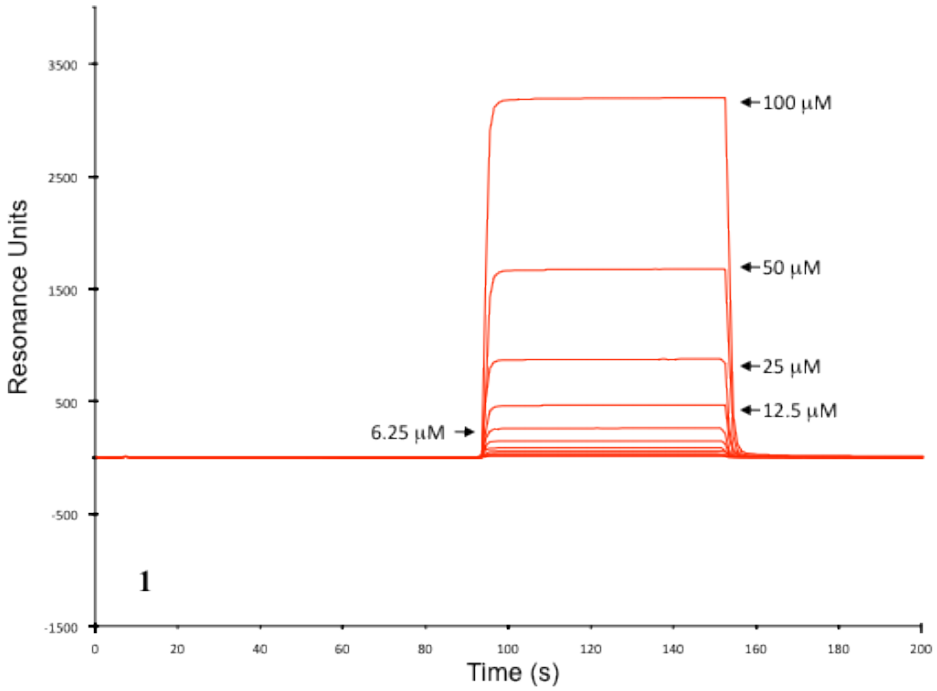


Figure S5. Example of typical SPR sensorgrams; in this case showing binding and unbinding events for **1** binding to c-kit2 oligonucleotide in the concentration range 0 – 100 μM. Ligand concentrations below those illustrated were: 3.125, 1.56, 0.78, 0.39, 0.19 0.1 and 0 μM.

		Expression Values			
Time	Treatment	Expt 1	Expt 2	Expt 3	Mean
4 hrs	Control	1.00	1.00	1.00	1.00
	1	-1.53	-1.68	-2.01	-1.48
	2	-3.23	-2.89	-4.88	-3.15
8 hrs	Control	1.00	1.00	1.00	1.00
	1	-2.02	-2.09	-3.78	-2.6
	2	-2.91	-2.44	-3.61	-3.44
24 hrs	Control	1.00	1.00	1.00	1.00
	1	-2.92	-3.82	-2.80	-2.87
	2	-2.58	-1.95	-2.20	-2.63

Table S1. Analysis of *c-KIT* mRNA expression levels in HCG-27 cells after small molecule treatment as assessed by qPCR. Fold change in *c-KIT* expression at different times after treatment with control (DMSO), **1** or **2**. The mean fold relative to expression of 3 housekeeping genes (GusB, UBC and YWHAZ) is expressed as the negative inverse of the fold change (n=3).

Ligand	K_d/μM									
	c-kit1	n	c-kit2	n	cMYC	n	hTelo	n	dsDNA	n
1	9.6 ± 4	1	1.0 ± 0.3	1	1.9 ± 0.4	1	3.6 ± 1.5	1	9.8 ± 1.7	1
2	8.3 ± 0.1	1	1.1 ± 0.3	1	4.3 ± 3	2	4.6 ± 1	2	n/s	-

Table S2. Biophysical analysis by Surface Plasmon Resonance of small molecule binding to G-quadruplex DNA sequences. Equilibrium dissociation constants for small molecule binding to c-kit1, c-kit2, cMYC, hTelo and dsDNA oligonucleotides in μM as determined from SPR data. (No specific interactions with double stranded DNA up to 50 μM for **2**). n indicates the calculated stoichiometry).

Materials and Methods

Cloning of minimal c-KIT core promoter

The *c-KIT* promoter region was taken as the region -124 to transcription start site (-1/+1). The *c-KIT* insert was constructed in a four-part ligation (see Figure S3), to minimize any secondary structure formation and inserted into the commercially available *pGL4.10* vector (Promega, Southampton, UK) at *Acc65I* and *EcoRV* restriction sites (New England Biolabs, Hitchin, UK).

Oligonucleotides

Deoxyoligonucleotide inserts (AccEcoTop1-3 and AccEcoBOT2; 5′ phosphorylated, HPLC purified) were synthesized by Biomers.net GmbH (Ulm, Germany).

AccEcoTop1:GTACCGCCGGGAAGAAGCGAGACCCGGGCGGGCGCGAGGGAGG,

AccEcoTop2:GGAGGCGAGGAGGGGCGTGGCCGGCGCGCAGAGGGAGGGCGCTG,

AccEcoTop3:GGAGGAGGGGCTGCTGCTCGCCGCTCGCGGCTCTGGGGGCTCGAT,

AccEcoBOT2:ATCGAGCCCCCAGAGCCGCGAGCGGCGAGCAGCAGCCCCTCCTCCCAGCGCCCTCCC TCTGCGCGCCGGCCACGCCCCTCCTCGCCTCCCCTCCCTCGCGCCCGCCCGGGTCTCGCTTCTTCCCG GCG.

Prior to use, deoxyoligonucleotides were resuspended in TE buffer (10 mM Tris.HCl, pH 7.4, 1 mM EDTA) to a concentration of 100 μM.

Annealing of c-KIT minimal promoter

The oligonucleotide mixture (12.5 μM of each, 200 μL total volume TE buffer plus 10 mM KCl) was annealed overnight (95°C for 5 min followed by slow cooling to RT), products were analysed by gel electrophoresis and the yield was estimated to be approximately 25% of starting materials. No further purification steps were required.

Enzyme digestion and gel purification of pGL4.10 plasmid

pGL4.10 plasmid (5 μg, Promega, Southampton, UK) was digested with *EcoRV* (50 U, 100U/μL stock; New England Biolabs, Hitchin, UK) and *Acc65I* (50 U, 10 U/μL stock; New England Biolabs) in a total volume of 100 μL (NEB Buffer 3, BSA; in accordance with supplier's protocol) at 37°C for 3 h. The mixture was heat denatured at 80°C for 20 min. Digested DNA was separated by gel electrophoresis and purified using a QIAquick Gel Extraction kit (Qiagen, Crawley, UK).

Ligation of c-KIT minimal promoter

12 ng of digested, gel purified *pGL4.10* backbone and 38 ng of annealed *c-KIT* insert (total of 50 ng DNA) were ligated at room temperature for 15 min with Quick-Stick Ligase (Bioline, London, UK) in a total of 20 μL (in accordance with supplier's instructions). 2 μL of the ligation mixture was used to transform 50 μL DH5α competent cells (library efficiency; Invitrogen, Paisley, UK). Clones were plated out onto 2×YT/agar plate (containing ampicillin at 100 μg/mL) and incubated at 37°C for 16 h.

Plasmid purification

Single colonies were picked and grown overnight (37°C, 16 h) in 10 mL LB broth (100 μg/mL ampicillin), plasmid DNA was extracted as per supplied protocol (Qiagen) and sequenced (Lark Technologies, Takeley, UK). Correctly sequenced plasmid was grown at a larger scale, purified (Midi/Maxi plasmid kit, Qiagen), resuspended in TE buffer and stored at -20°C prior to transfection optimisation.

Surface Plasmon Resonance

The oligonuclotides used for Surface Plasmon Resonance equilibrium binding experiments were:

c-kit1: d(biotin[AGGGAGGGCGCTGGGAGGAGGG],

c-kit2: d(biotin[CCCGGGCGGGCGCGAGGGAGGGGAGG]),

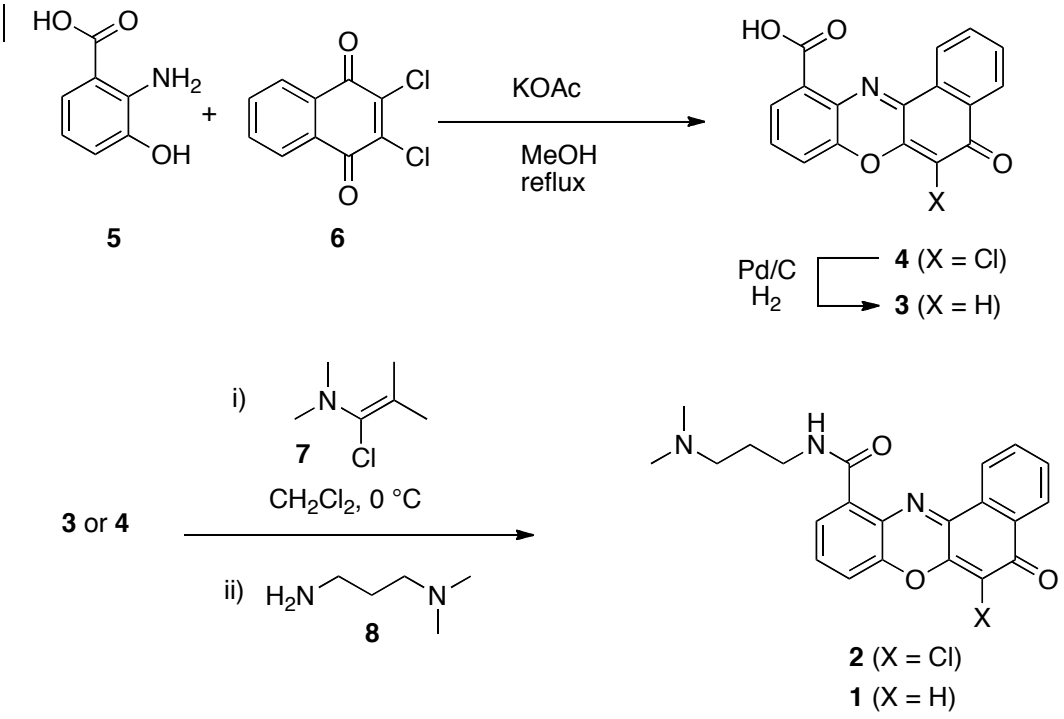
c-MYC: d(biotin[TGGGGAGGGTGGGGAGGGTGGGGAAGG]),

hTelo: d(biotin[GTTA$(GGGTTA)_4$GG]),

and double stranded DNA (dsDNA) comprising the oligonucleotide d(biotin[GGCATAGTGCGTGGGCGTTAGC]) hybridized with its complementary strand.

Preparation of Substituted Benzo[*a*]phenoxazines:

General methods: All starting materials were obtained from commercial suppliers and used as received. Solvents were purified by standard techniques. Experiments were carried out under inert atmosphere. ^{1}H NMR and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz using a Bruker DRX 500 instrument. ^{1}H spectra were recorded in deuterated solvents as detailed and at ambient probe temperature (300 K). Chemical shifts are reported in parts per million (ppm) and are referenced to the residual solvent peak. The following notations are used: singlet (s); doublet (d); triplet (t); quartet (q); multiplet (m); broad (br). Data are reported in the following manner: chemical shift (multiplicity, coupling constant if appropriate, integration). Signals are quoted as δ values in ppm and coupling constants (J) are reported in Hertz. Absorption spectra were recorded on a Cary 400 UV/Vis spectrophotometer. Mass spectra were recorded on a Micromass Q-Tof (ESI) spectrometer. HPLC was performed using a Varian Pursuit C18, 5μ column (250 × 21.2 mm) and a gradient elution with 0.1% TFA/H_2O (solvent A) and 0.1% TFA/MeCN (solvent B) at a flow rate of 12.0 mL/min.



Scheme S1

Description of syntheses: Benzo[*a*]phenoxazines **1-4** were synthesized (Scheme S1) using a procedure derived from that described by Agarwal, *et al*.[17] Benzo[*a*]phenoxazine carboxylic acid derivative **4** was prepared by refluxing 3-hydroxyanthranilic acid (**5**) and 2,3-dichloro-1,4-naphthoquinone (**6**) with potassium acetate in methanol.[16] The carboxylic acid **4** was dechlorinated by hydrogenation to give benzo[*a*]phenoxazine derivative **3**. Both carboxylic acids **3** and **4** were activated by using the Ghosez reagent (**7**) to the corresponding acid chlorides *in situ* and were coupled with *N*,*N*-dimethyl-1,3-propanediamine (**8**) to provide **1** and **2** respectively.

Benzo[*a*]phenoxazine carboxylic acid (4): 3-hydroxyanthranilic acid (**5**, 250 mg, 1.60 mmol), 2,3-dichloro-1,4-naphthoquinone (**6**, 420 mg, 1.60 mmol) and potassium acetate (310 mg, 3.20 mmol) were heated to reflux in methanol overnight and allowed to cool to RT and then in ice. The resultant precipitate was filtered and washed with water and ice-cold methanol, dried over P_2O_5 then recrystallised from MeOH:CH_2Cl_2. The compound was obtained as deep red needles (460 mg, 80%). ^{1}H-NMR (500 MHz, 7:1 C_6D_6: DMSO-d_6): 8.73-8.70 (*m*, 1H), 8.44-8.42 (*m*, 1H), 7.86 (*dd*, 1H, J = 5.7, 1.6 Hz), 7.51-7.49 (*m*, 2H), 7.21-7.16 (*m*, 2H); ^{13}C-NMR (125 MHz, 7:1 C_6D_6: DMSO-d_6): 176.9, 167.4, 146.5, 146.5, 143.5, 132.8, 132.6, 132.5, 131.7, 131.3, 130.5, 130.3, 126.6, 126.4, 125.4, 118.6, 115.0. HRMS (ESI): Calculated for $C_{17}H_7NO_4Cl$ ([M-H]$^-$): 324.0069. Found: 324.0069.

Benzo[*a*]phenoxazine carboxylic acid (3): Carboxylic acid **4** (80 mg, 0.24 mmol) and Pd (10% on Carbon, 10 mg) were stirred in MeOH (20 mL) and DMF (20 mL) under a hydrogen atmosphere overnight at room temperature overnight. The solution was filtered through Celite® and the solvent removed *in vacuo*. The resulting orange solid was dissolved in CH_2Cl_2 (20 mL), washed with water (5 × 20 mL) and dried over $MgSO_4$. The solvent was removed to afford **4** as an orange solid (60 mg, 94%). The product was used without further purification.

Preparation of 1 and 2:

General procedure: Carboxylic acid derivative **3** or **4** (1 eq) was suspended in anhydrous CH_2Cl_2 and cooled to 0 °C in an argon atmosphere. 1-Chloro-*N*,*N*-2-trimethyl-1-propenyl amine (**7**) (2-5 eq) was added and allowed to stir until the solution became clear. Then triethylamine (> 10 eq) was then added, followed by amine **8** (3 eq). The solution was then allowed to warm to RT and stirred overnight, dried *in vacuo* and directly purified by prep-HPLC to give carboxamide derivatives **1** and **2**.

Benzo[*a*]phenoxazine carboxamide (2): According to the above procedure **2** was prepared using **4** (100 mg, 0.30 mmol), 1-chloro-*N*,*N*-2-trimethyl-1-propenyl amine, **7** (500 μL, 3.73 mmol), triethylamine (2 mL, 14.46 mmol) and *N*,*N*-dimethyl-1,3-propanediamine, **8** (112 μL, 0.90 mmol). HPLC (gradient: 10 to 80% MeCN/water over 22 min, R_t = 18.5 min). **1** was obtained as an orange solid (123 mg, 98%). ^{1}H-NMR (500 MHz, DMSO-d_6): 10.28 (*br s*, 1H), 8.95 (*t*, 1H, *J* = 5.6 Hz), 8.57 (*br d*, 1H, *J* = 7.7 Hz), 8.27 (*br d*, 1H, *J* = 7.7 Hz), 8.06 (*t*, 1H, *J* = 7.3 Hz), 7.96 (*t*, 1H, *J* = 7.3 Hz), 7.76-7.70 (*m*, 3H), 3.57-3.51 (*m*, 2H), 3.16 (*t*, 2H, *J* = 7.9 Hz), 2.78 (*s*, 6H); 2.07-2.04 (*m*, 2H); ^{13}C-NMR (125 MHz, DMSO-d_6): 176.7, 165.3, 147.1, 146.1, 143.2, 135.1, 133.2, 132.8, 131.9, 130.9, 129.9, 129.4, 126.1, 125.9, 125.0, 118.0, 112.8, 54.8, 43.3, 36.7, 24.7; HRMS (ESI): Calculated for $C_{22}H_{21}N_3O_3Cl$ ($[M+H]^+$): 410.1271. Found: 410.1274.

Benzo[*a*]phenoxazine carboxamide (1): According to the above procedure **1** was prepared using **3** (50 mg, 0.17 mmol), 1-chloro-*N*,*N*-2-trimethyl-1-propenyl amine (**7**, 100 μL, 0.74 mmol), triethylamine (1 mL, 7.23 mmol) and *N*,*N*-dimethyl-1,3-propanediamine (**8**, 60 μL, 0.51 mmol). HPLC (gradient: 10 to 70% MeCN /water over 22 min, R_t = 15.0 min). **2** was obtained as an orange solid (66 mg, 60%). ^{1}H-NMR (400 MHz, CD_3OD): 8.62 (*dd*, 1H, *J* = 7.7, 1.4 Hz), 8.29 (*dd,* 1H, *J* = 7.4, 1.6 Hz); 7.95-7.88 (*m*, 3H), 7.70 (*t,* 1H), 7.60 (*dd,* 1H, *J* = 8.2, 1.4 Hz), 6.50 (*s,* 1H), 3.74 (*t*, 2H, *J* = 6.7 Hz), 3.37-3.32 (*m*, 2H), 2.98 (*s*, 6H); 2.25-2.18 (*m*, 2H); ^{13}C-NMR (100 MHz, CD_3OD): 185.5, 168.9, 152.8, 149.1, 145.4, 134.2, 133.8, 132.7, 133.5, 132.7, 132.2, 131.3, 127.2, 127.0, 125.9, 120.0, 108.2, 56.8, 43.6, 37.6, 26.5; HRMS (ESI): Calculated for $C_{22}H_{22}N_3O_3$ ($[M+H]^+$): 376.1656. Found: 376.1654.

Supplementary References:

(17) Agarwal, N. L.; Schafer, W. *J. Org. Chem.* **1980**, 45, 2155.

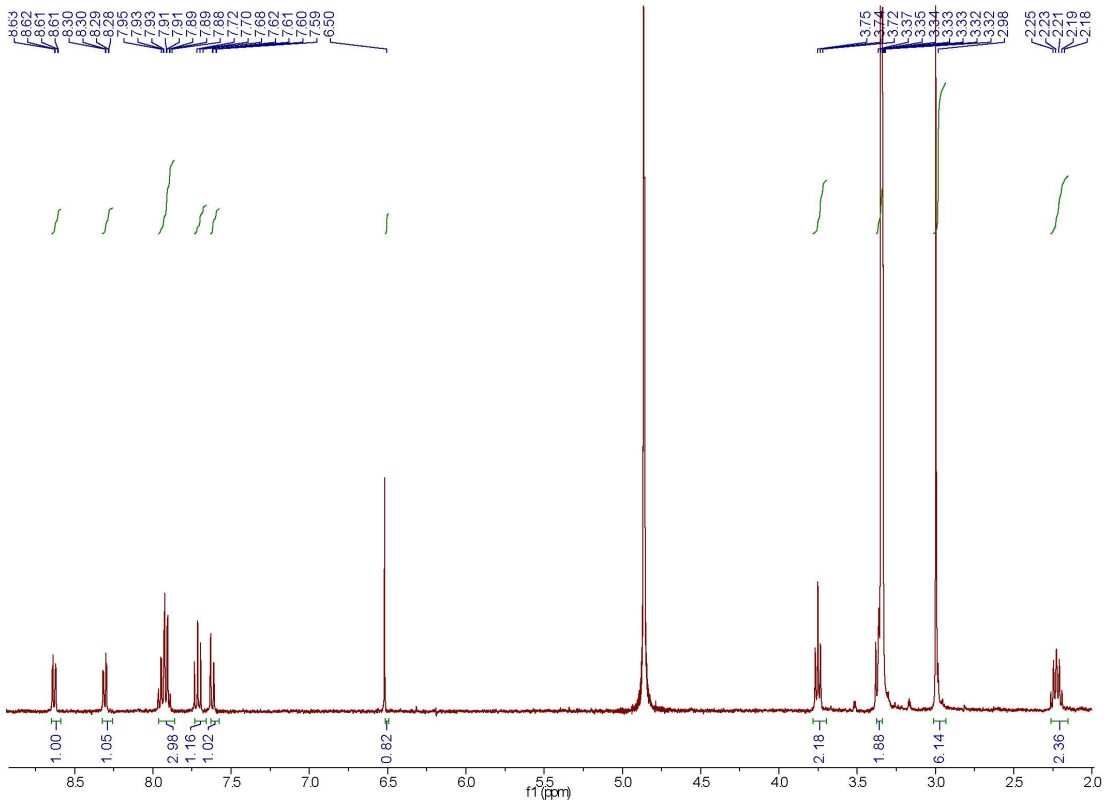


Figure S5. Illustrative ^{1}H-NMR spectrum for **1**

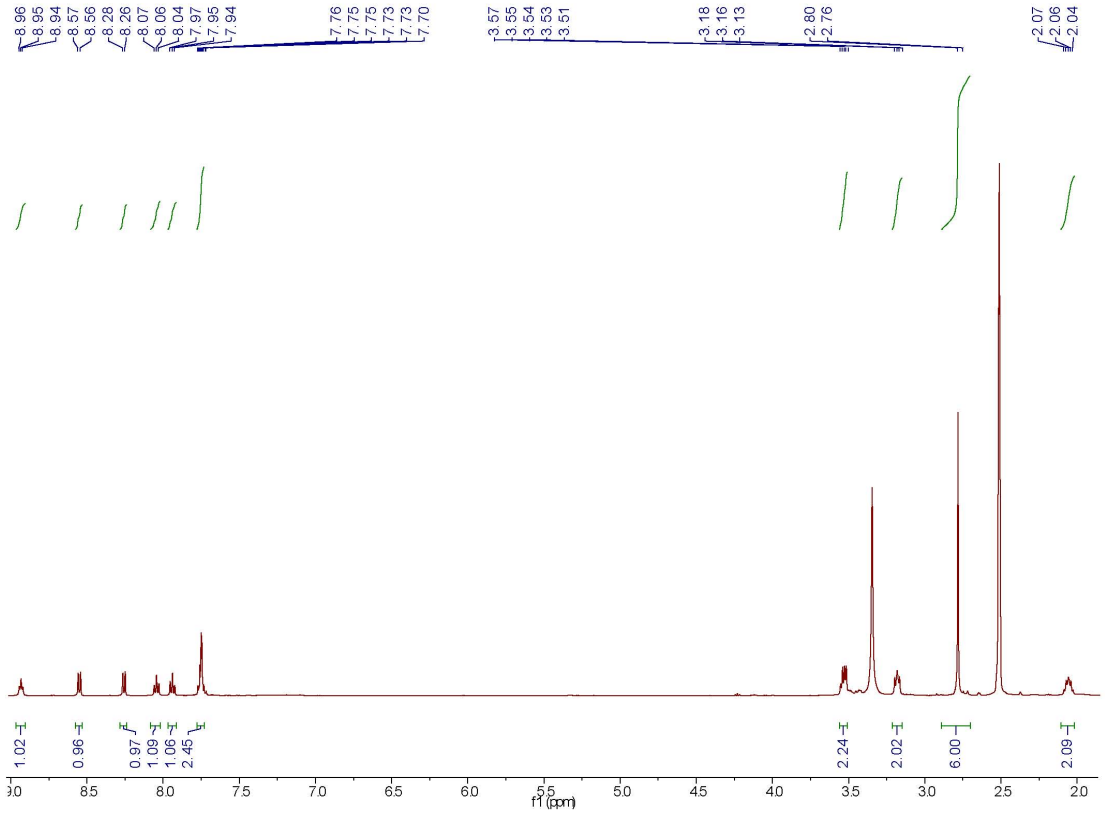


Figure S6. Illustrative ^{1}H-NMR spectrum for **2**.

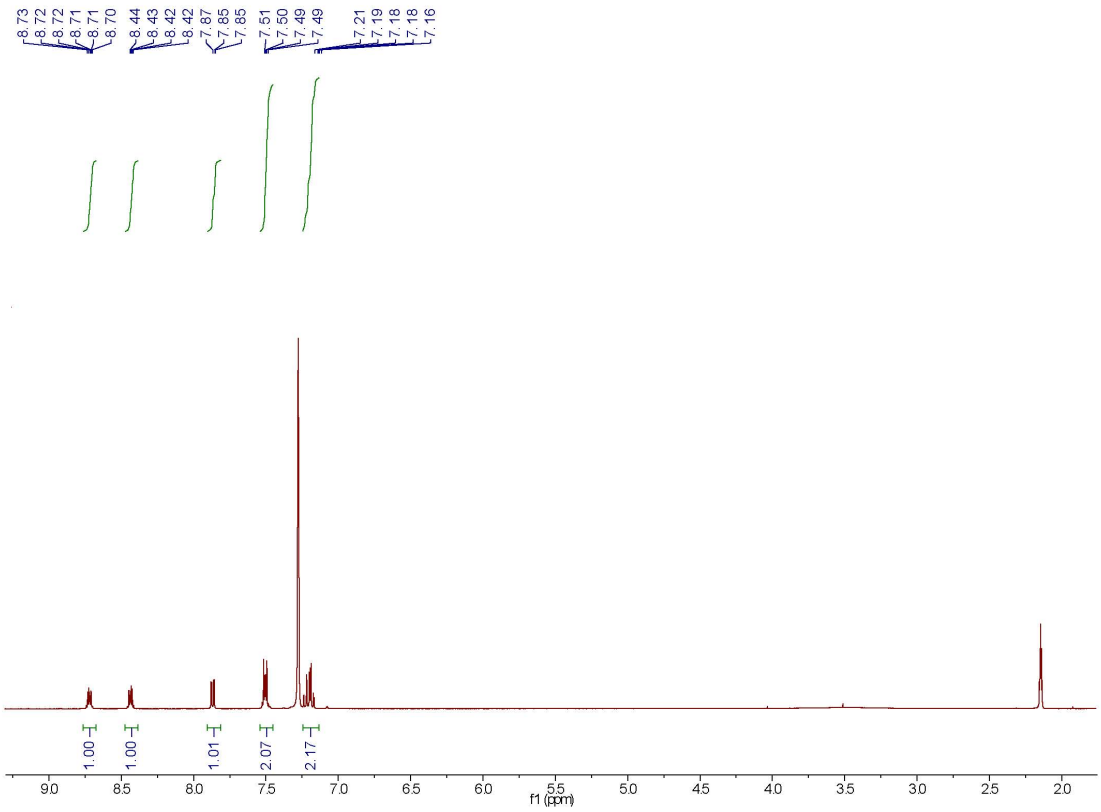


Figure S7. Illustrative ^{1}H-NMR spectrum for **4**.