Supporting Information

Inhibition of the HIF1α-p300 interaction by quinone- and indandione-mediated ejection of structural Zn(II)

Madura K. P. Jayatunga, Sam Thompson, Tawnya C. McKee, Mun Chiang Chan, Kelie M. Reece, Adam. P. Hardy, Rok Sekirnik, Peter T. Seden, Kristina M. Cook [c], James B. McMahon, William D. Figg,[a*] Christopher J. Schofield,[b*] and Andrew D. Hamilton[b*]

[a] *Center for Cancer Research, National Cancer Institute, Building 10, Bethesda, MD 20892-1906*

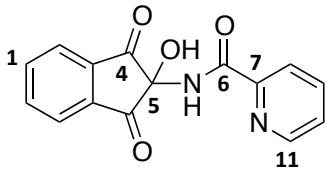
[b] *Department of Chemistry, Chemistry Research Laboratory, University of Oxford, Mansfield Road, Oxford, OX1 3TA, U.K.*

figgw@helix.nih.gov, christopher.schofield@chem.ox.ac.uk, andrew.hamilton@chem.ox.ac.uk

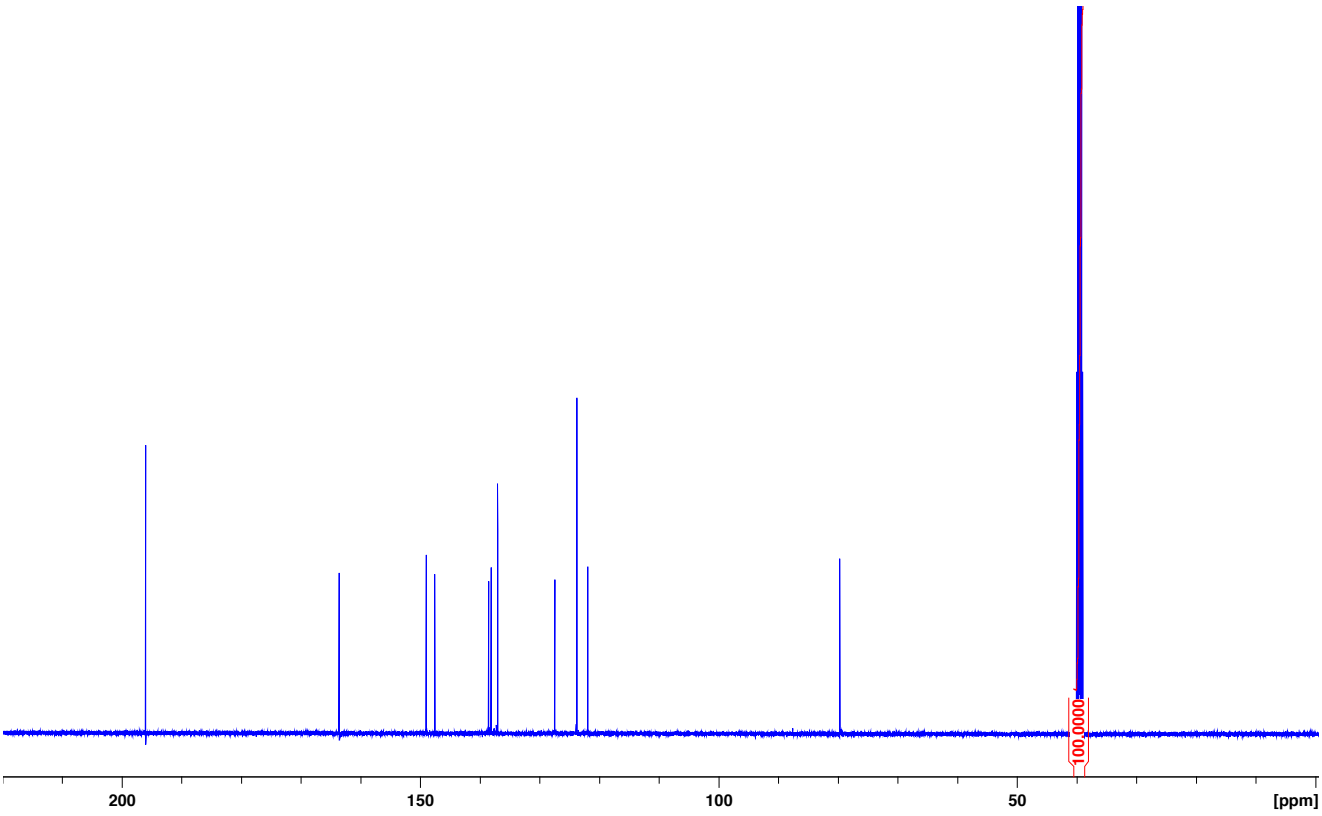
CONTENTS

1. NMR Spectra

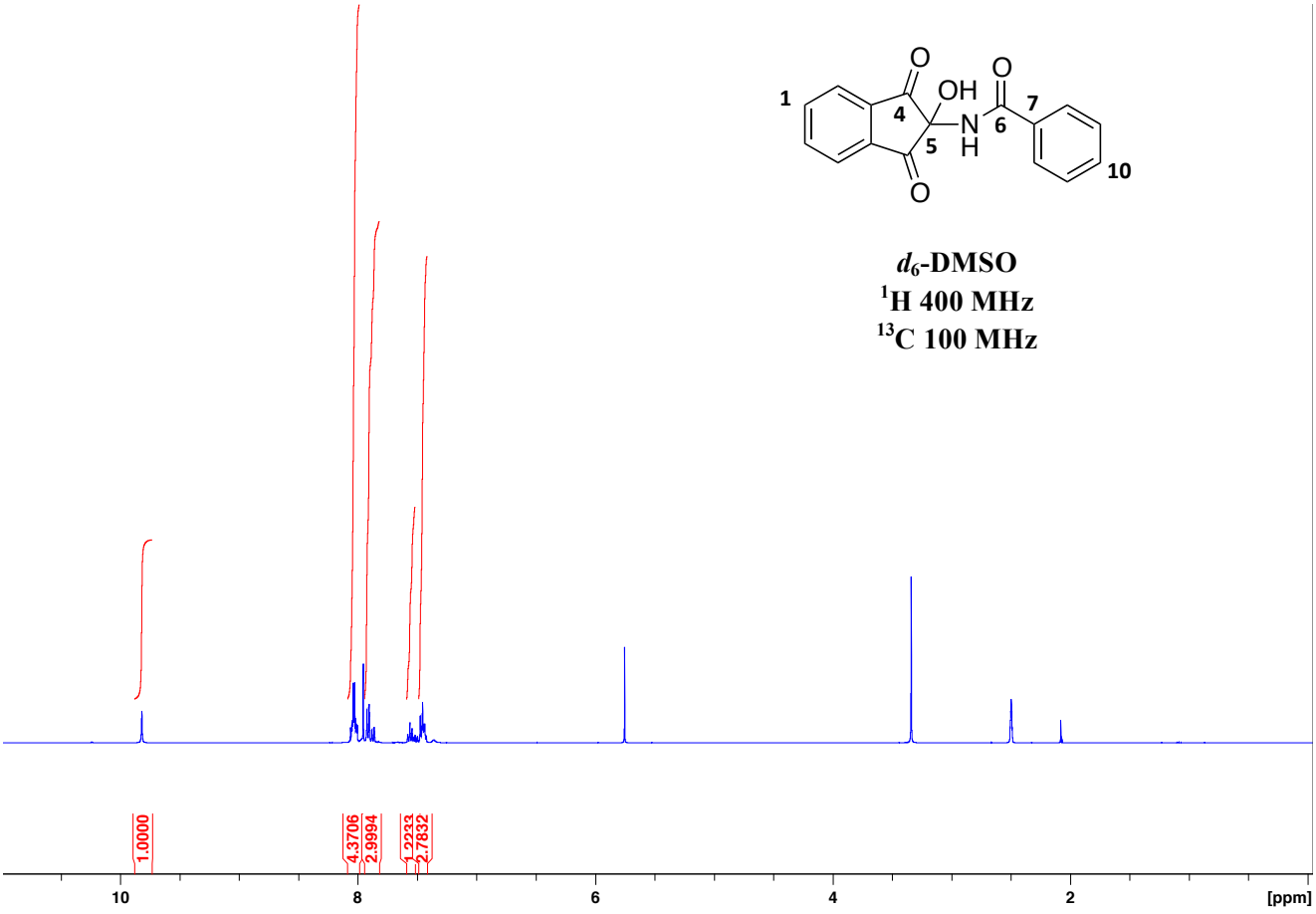
1.1 N-(2-Hydroxy-1,3-dioxo-2,3-dihydro-1H-inden-2-yl)picolinamide (5)

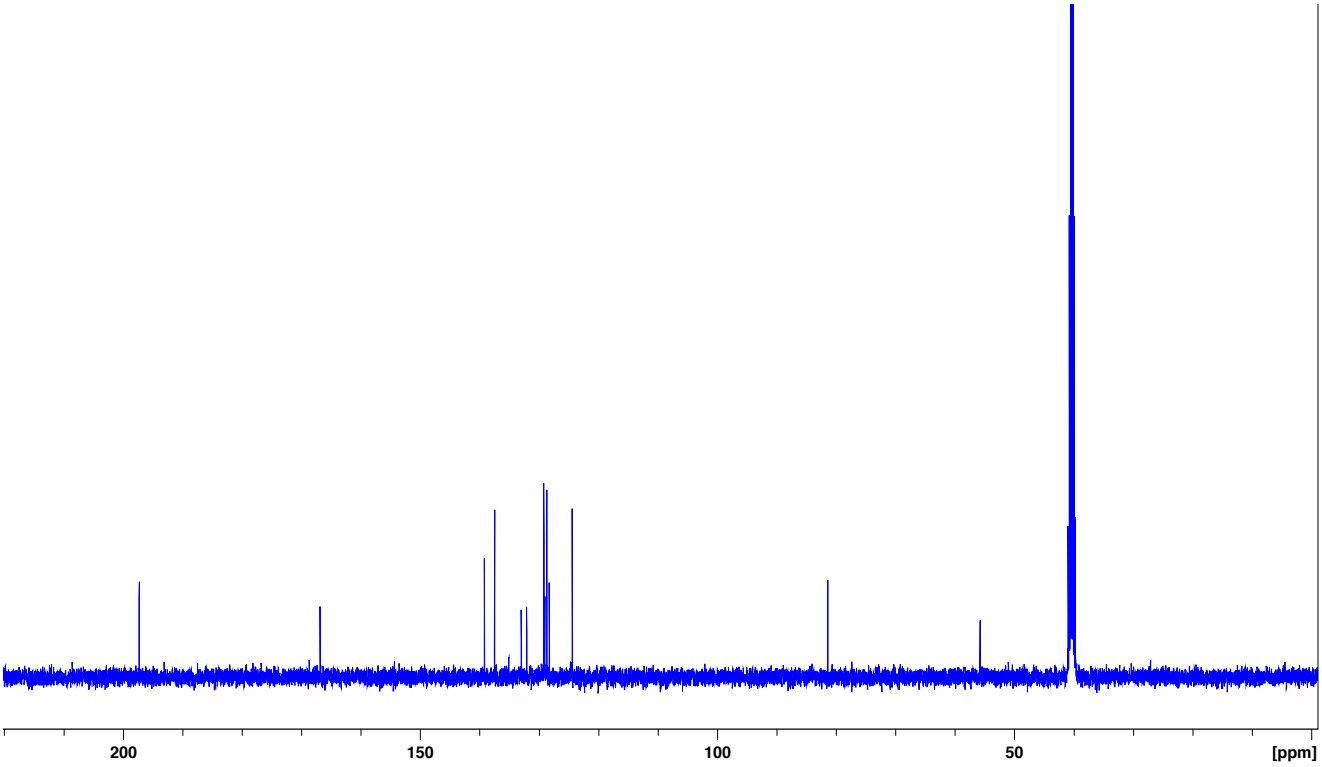


d_6-DMSO
^{1}H 500 MHz
^{13}C 125 MHz



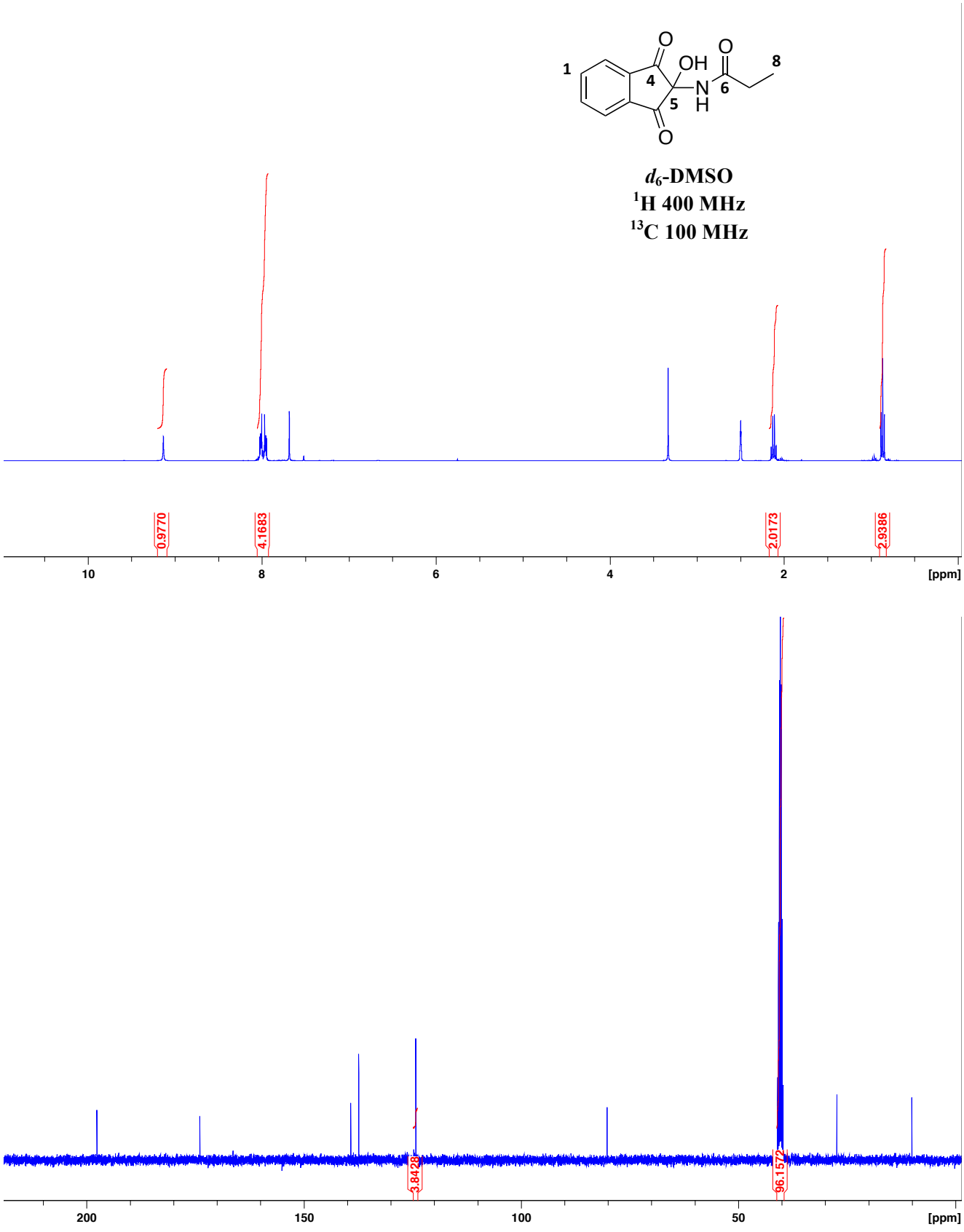
1.2 N-(2-Hydroxy-1,3-dioxo-2,3-dihydro-1H-inden-2-yl)benzamide (14)



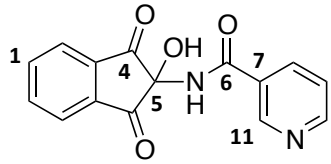


d_6-DMSO
^{1}H 400 MHz
^{13}C 100 MHz

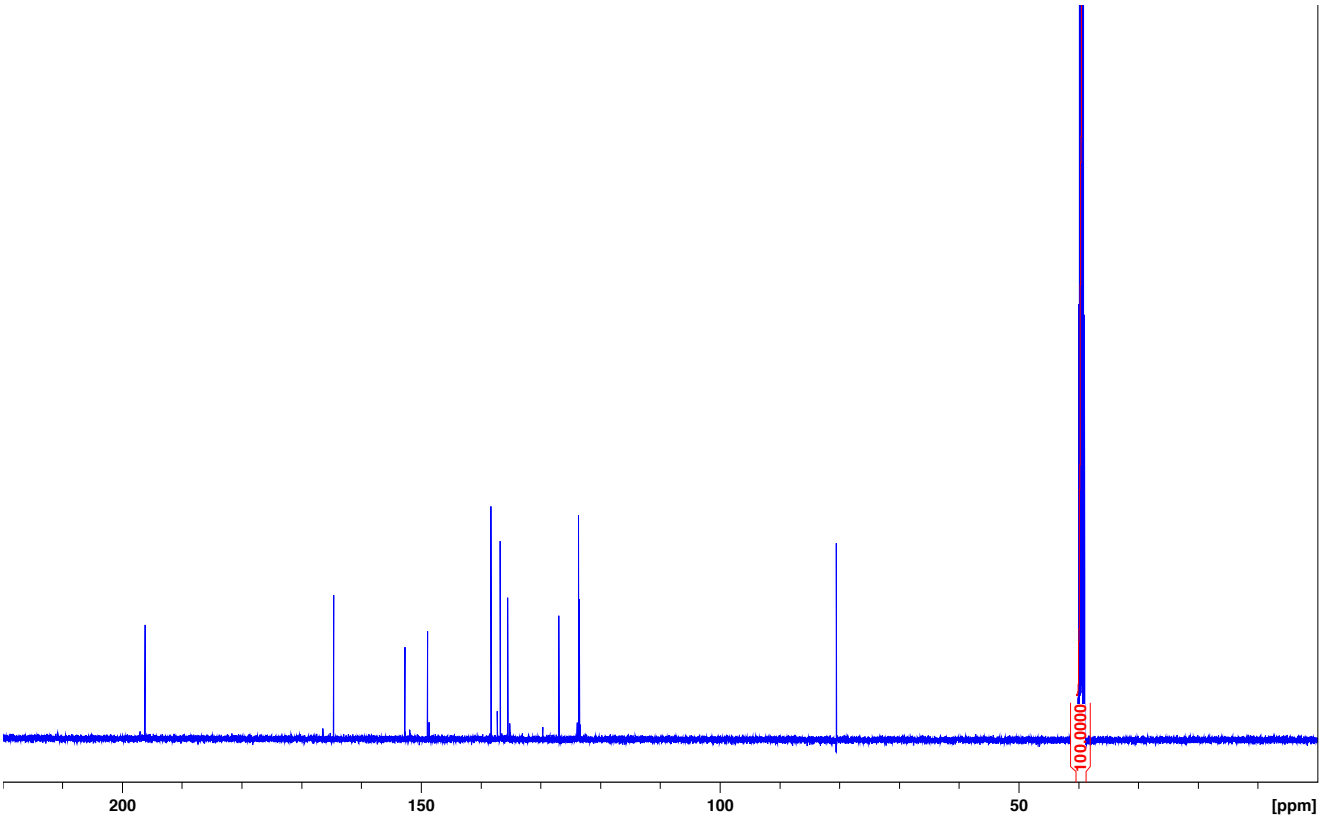
1.3 N-(2-Hydroxy-1,3-dioxo-2,3-dihydro-1H-inden-2-yl)propionamide (15)



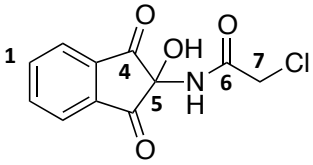
1.4 N-(2-Hydroxy-1,3-dioxo-2,3-dihydro-1H-inden-2-yl)nicotinamide (16)



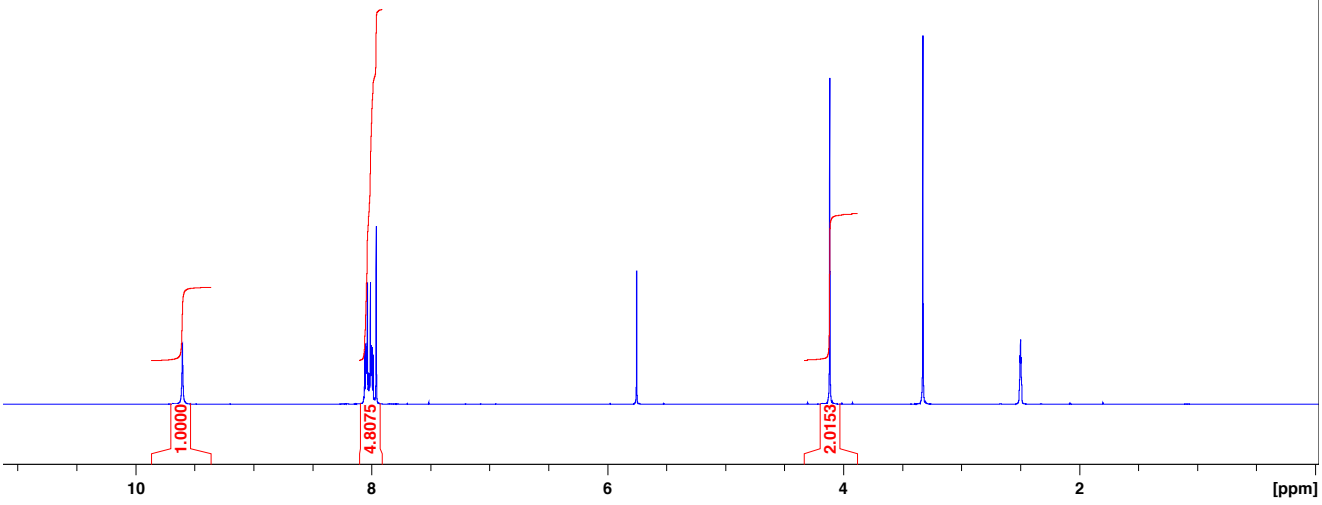
d_6-DMSO
^{1}H 500 MHz
^{13}C 125 MHz

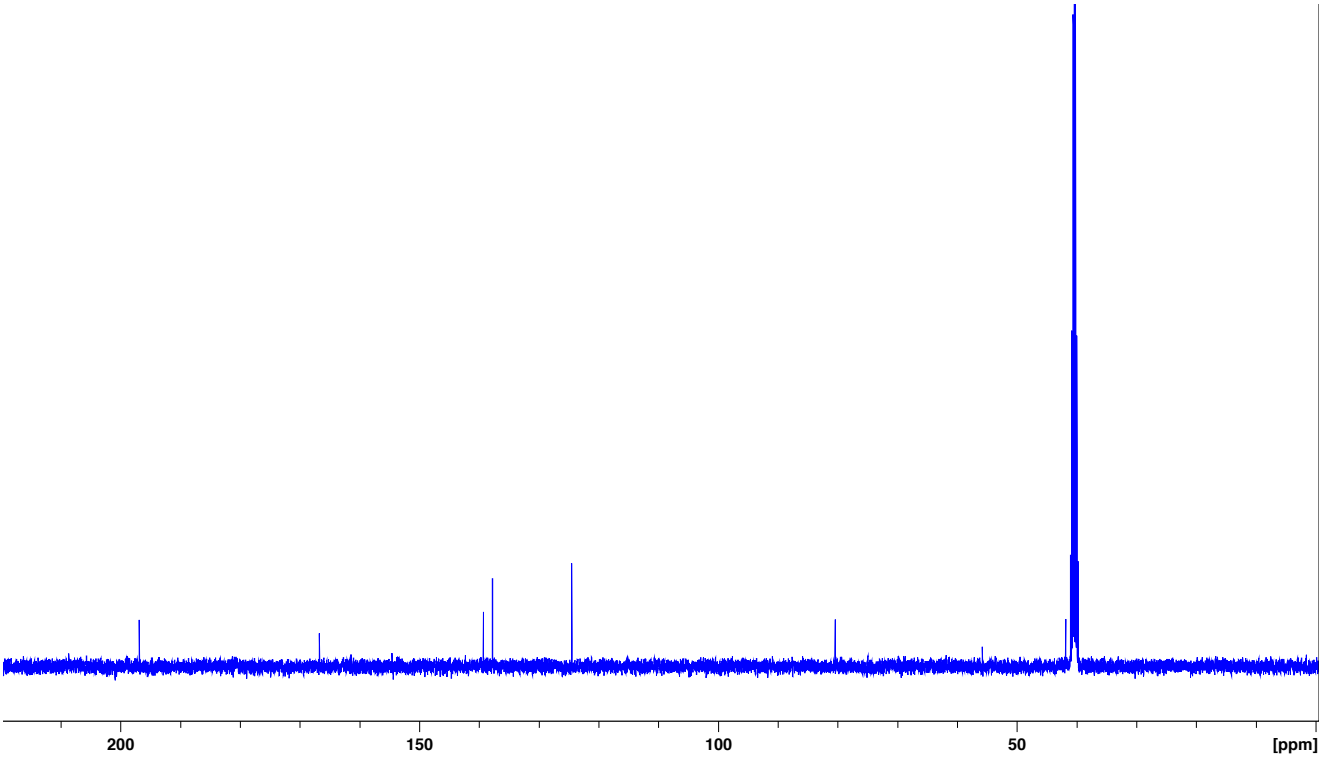


1.5 2-Chloro-N-(2-hydroxy-1,3-dioxo-2,3-dihydro-1H-inden-2-yl)acetamide (17)

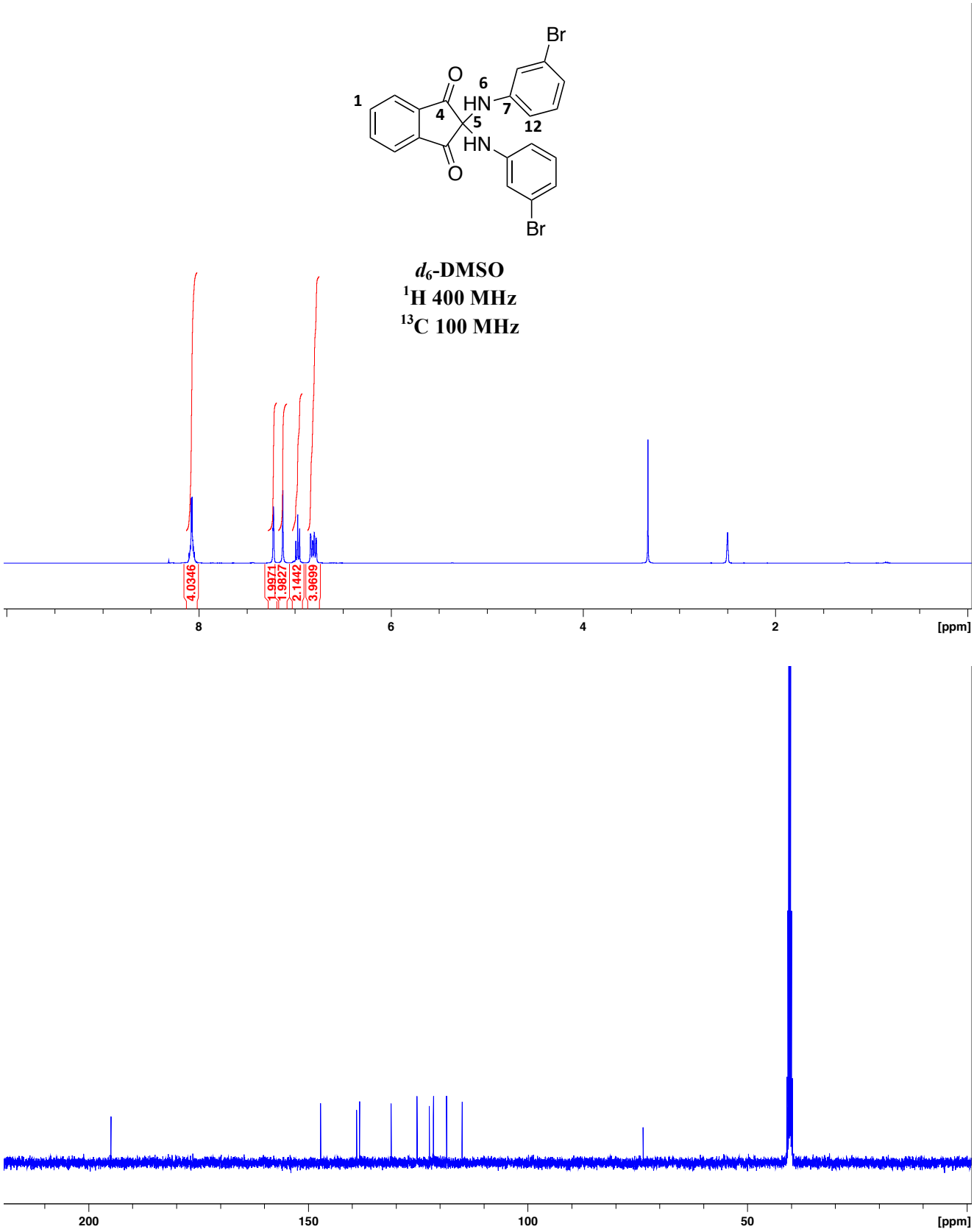


d_6-DMSO
^{1}H 400 MHz
^{13}C 100 MHz

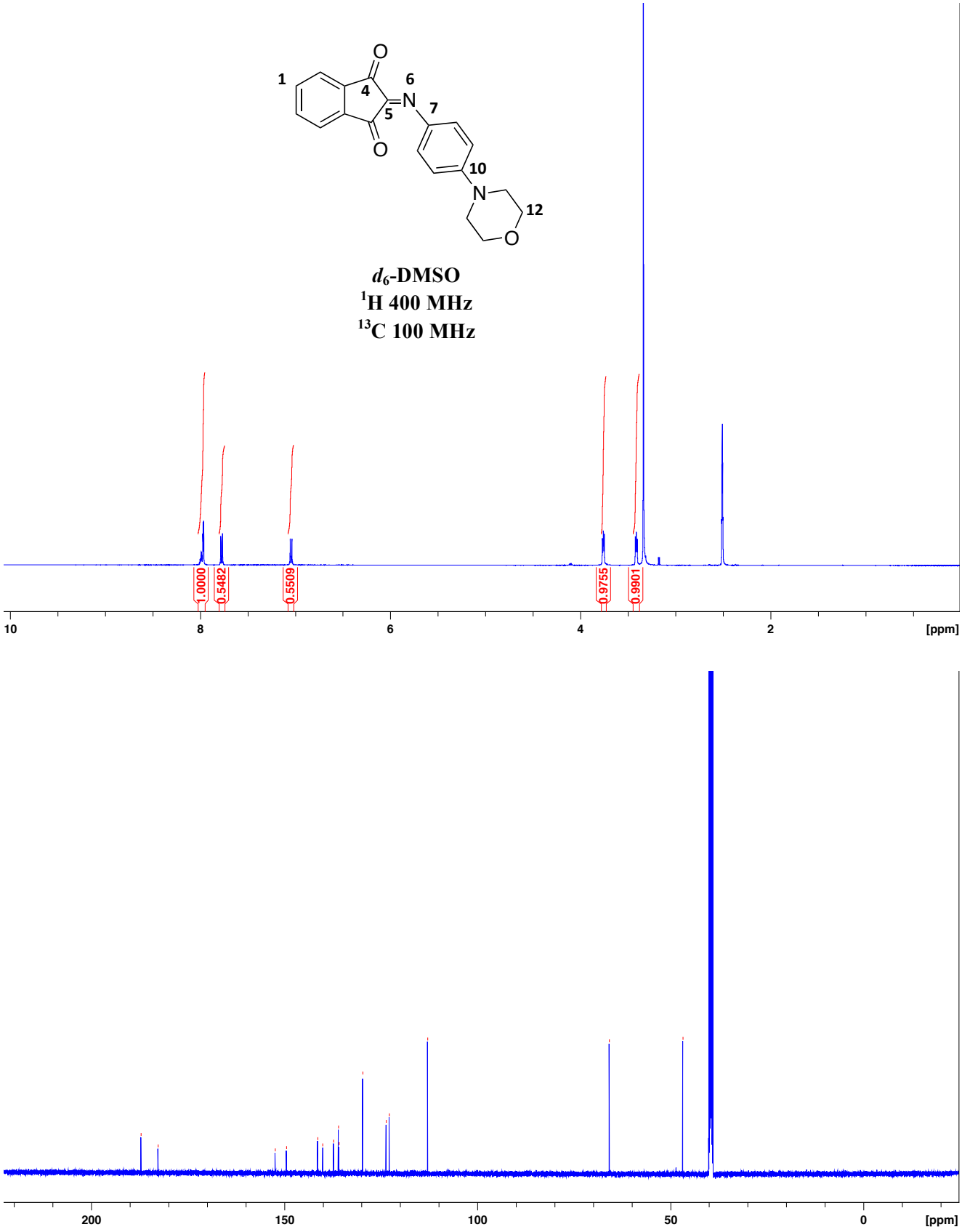




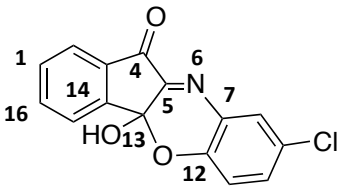
1.6 2,2-bis((3-Bromophenyl)amino)-1H-indene-1,3(2H)-dione (18)



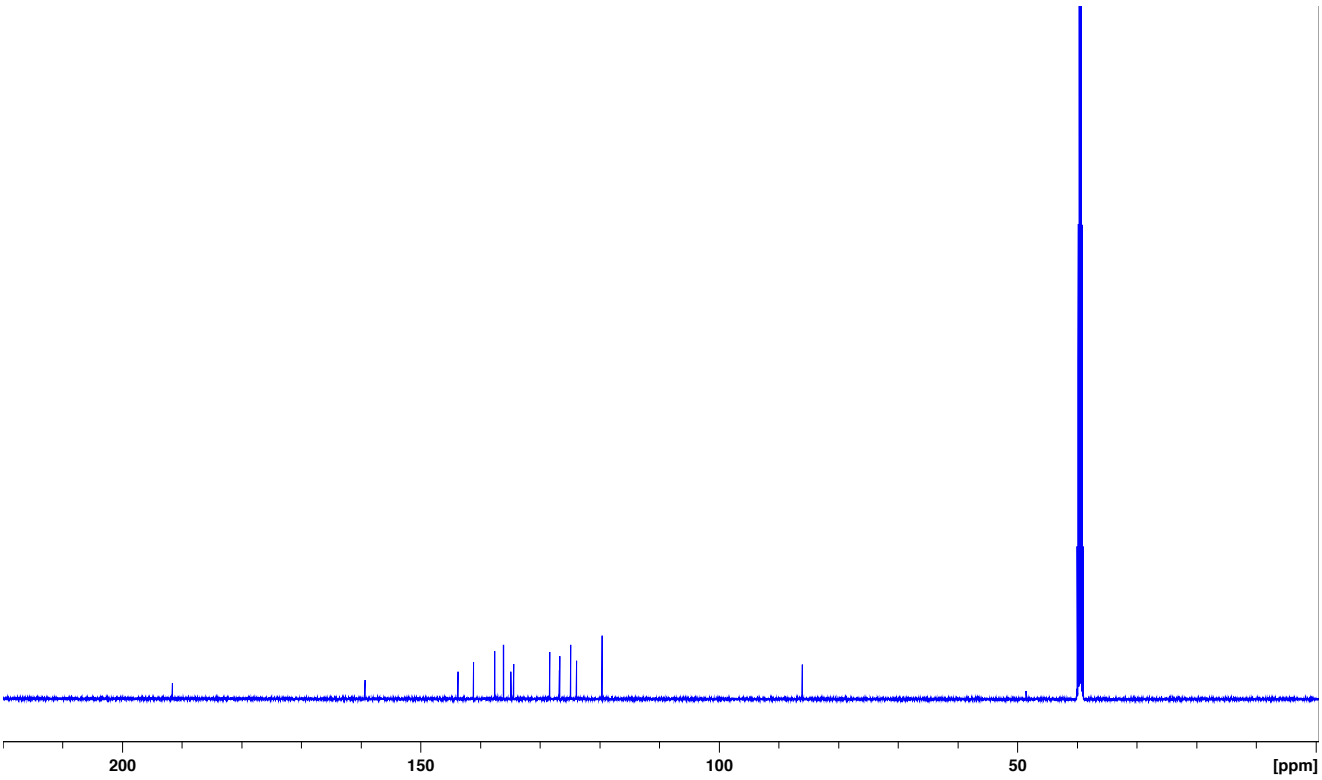
1.7 2-((4-Morpholinophenyl)imino)-1H-indene-1,3(2H)-dione (19)



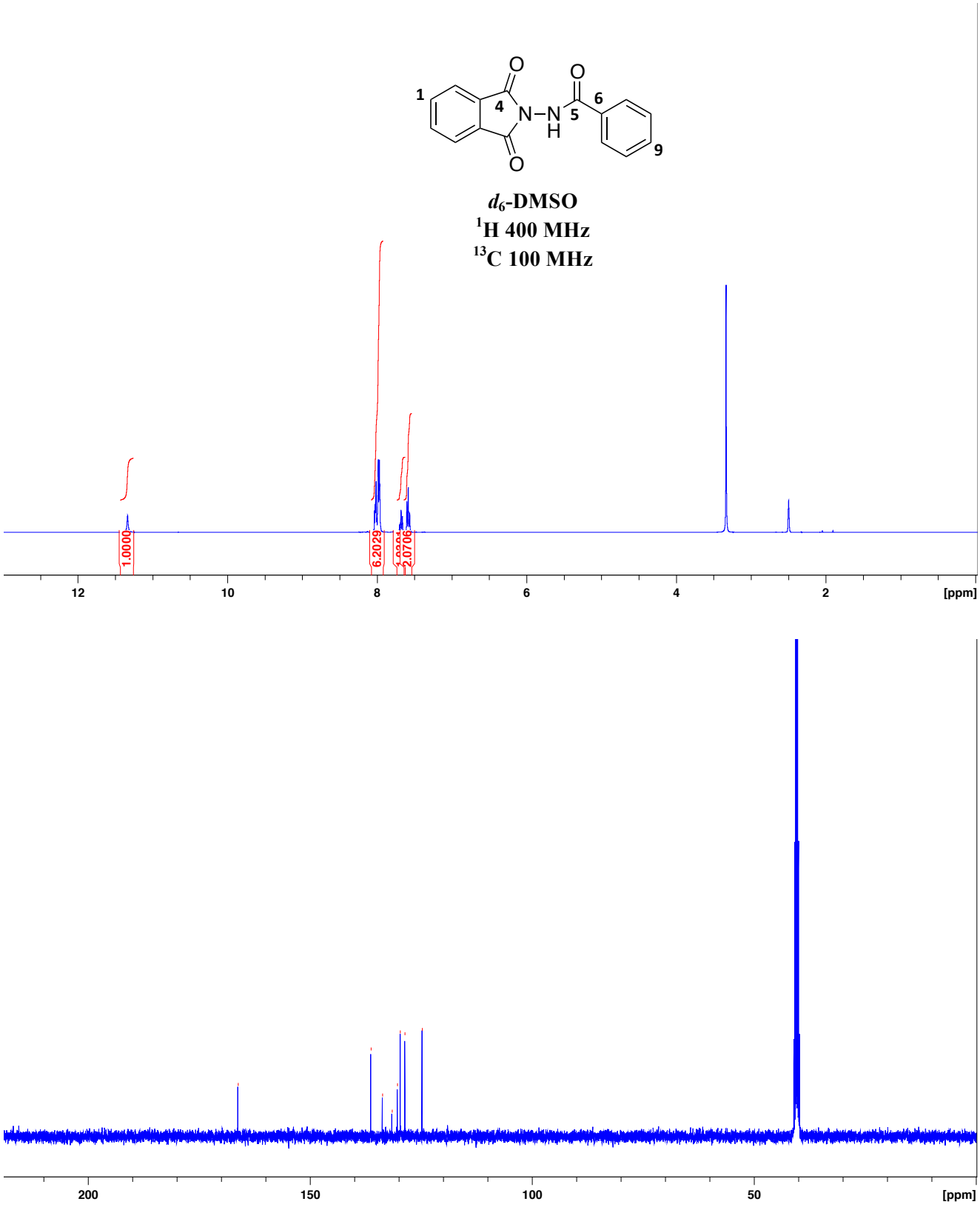
1.8 8-Chloro-4b-hydroxybenzo[b]indeno[2,1-e][1,4]oxazin-11(4bH)-one (20)



d_6-DMSO
^{1}H 500 MHz
^{13}C 125 MHz



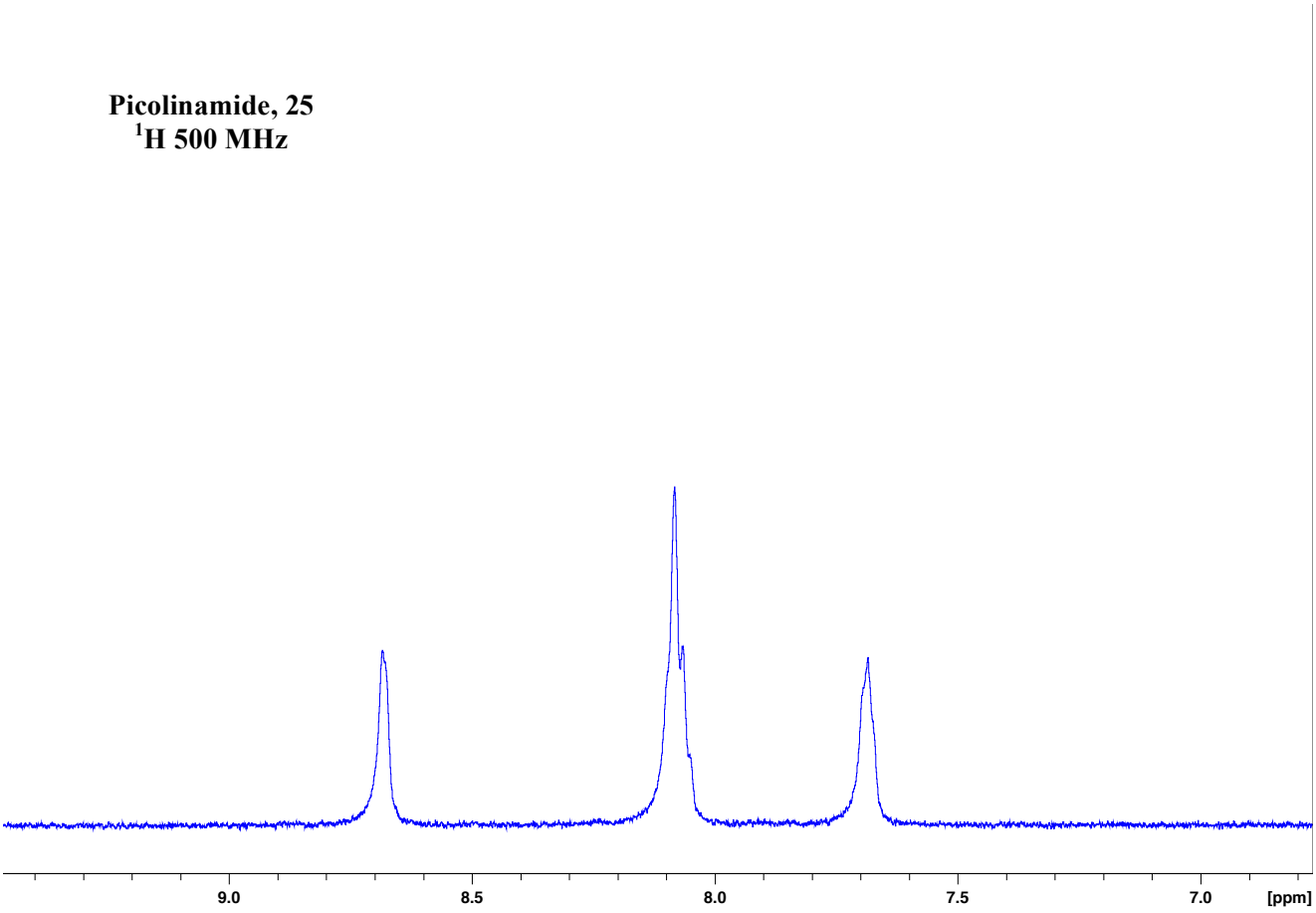
1.9 N-(1,3-Dioxoisoindolin-2-yl)benzamide (21)



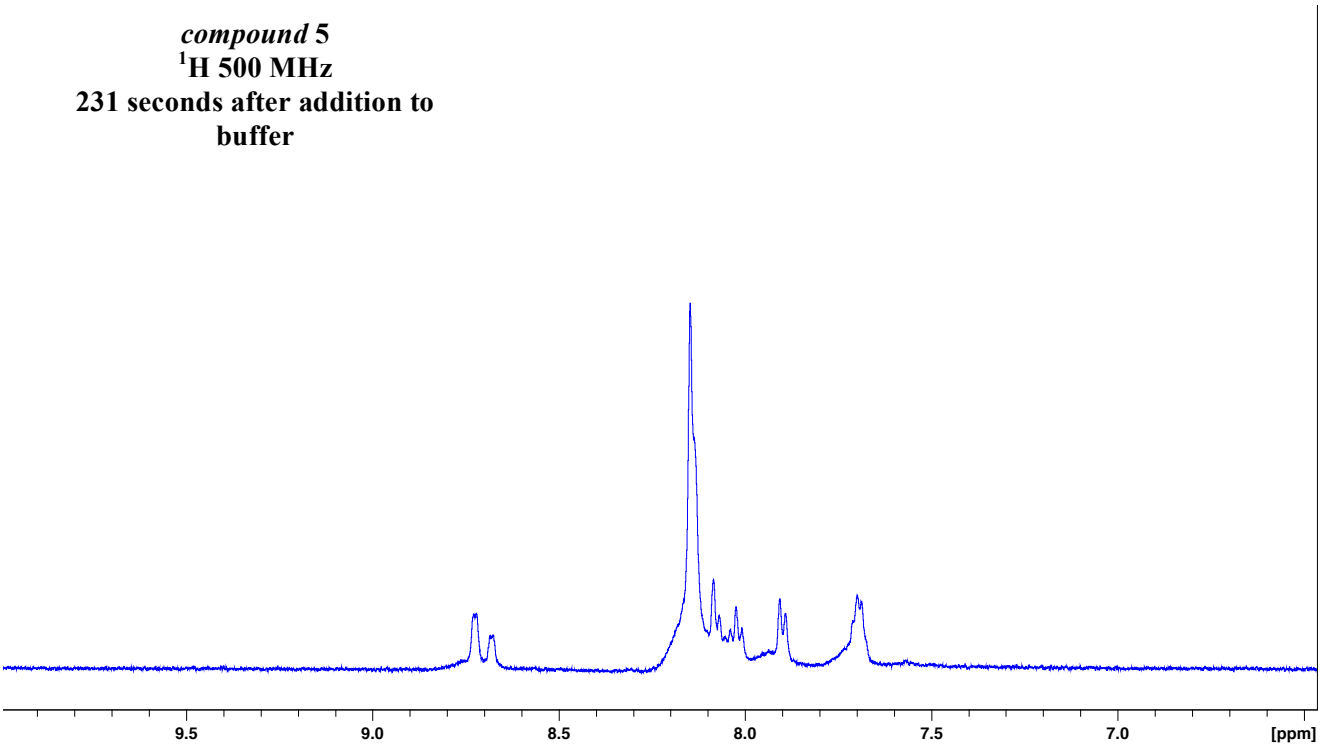
1.10 NMR Timecourse

NMR samples were prepared by adding solutions of compound (100 µL, 30 mM in d_6-DMSO) to deuterated phosphate buffer (500 µL, 10 mM, pH 8). As rapidly as possible after the addition 30 ^{1}H NMR experiments were run sequentially, with a three second delay between acquisitions.

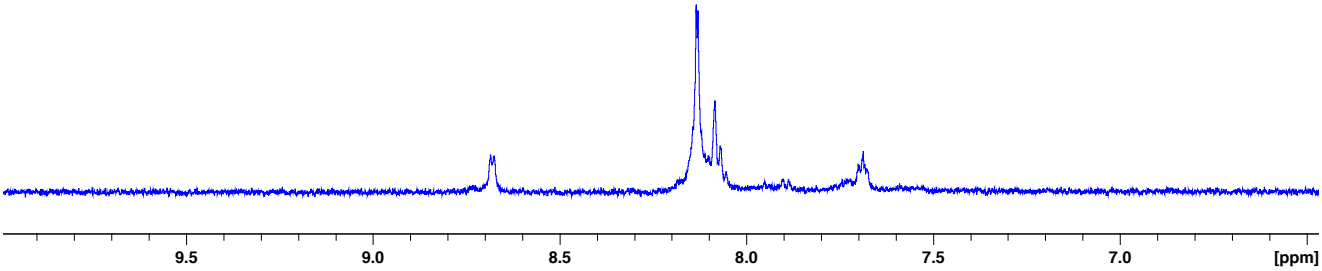
Picolinamide, 25
^{1}H 500 MHz

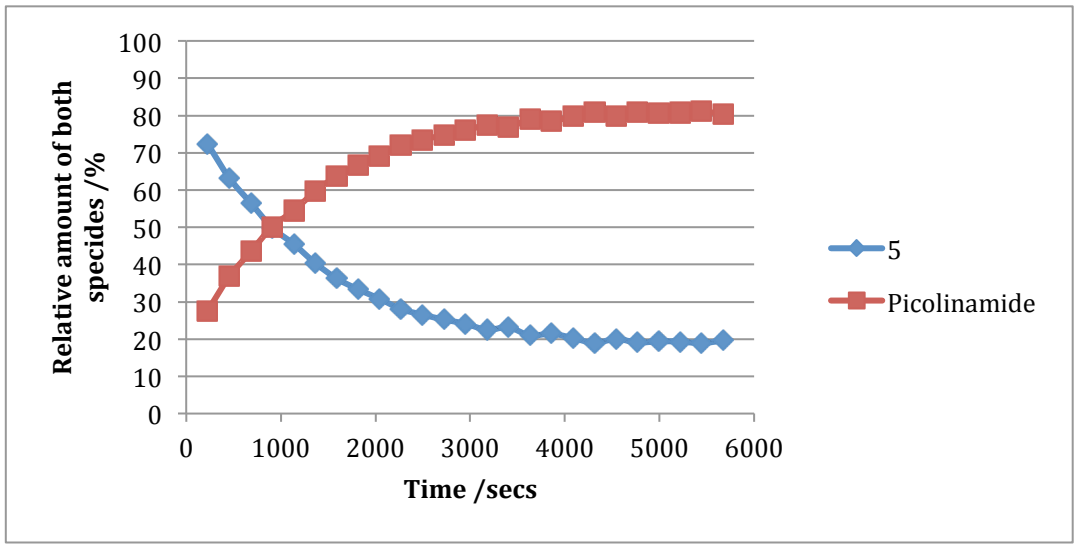


***compound* 5**
^{1}H 500 MHz
231 seconds after addition to buffer



***compound* 5**
^{1}H 500 MHz
2494 seconds after addition to buffer





2. Assays

2.1 Protein expression

Recombinant *N*-terminal GST-tagged $p300_{323-423}$ was overexpressed and purified as described for GST-tagged $p300_{302-423}$ by Kung *et al*.[1] The following modifications were made: purification used a glutathione-Sepharose 4B (GE Healthcare) column (20 mL) with further purification by gel filtration chromatography using a Superdex 200 column (GE Healthcare). Protein was stored in TBST buffer with 10 μM $ZnCl_2$, 0.5 mM DTT, and the protein concentration determined by Bradford assay.

Expression and purification of JMJD2A was performed according to the method of Ng *et al*.[2]

2.2 JMJD2A FluoZin^{TM}-3 Zn ejector Assay

Zinc ejection of compounds was measured by monitoring the concentration of Zn(II) *in situ* with the Zn-specific fluorophore FluoZin^{TM}-3 (FZ-3, Invitrogen, Figure S3), as reported by Sekirnik *et al*.[3] Before each experiment, a calibration curve (between 0-2 μM Zn(II)) was obtained with varied concentrations of $ZnCl_2$ dissolved in MilliQ water. For assays, 1 mM solution of FluoZin^{TM}-3 was prepared in 50 mM HEPES buffer, pH 7.5, and then diluted in the same buffer to 10 μM stock solution. 10 μL of the stock solution was pre-mixed with 10 μL of 20 μM JMJD2A enzyme and 30 μL buffer to make the enzyme mix. A solution of compound in DMSO (5 μL) was mixed with 45 μL buffer. The compound solution was then mixed with the enzyme-FZ-3 solution to give a total volume of 100 μL, with final concentrations of FluoZinTM-3, enzyme and DMSO of 1 μM, 2 μM and 5 %, respectively. A Novostar fluorescence spectrometer (BMG Lab technologies) was used for the measurements. Mixing of the enzyme mix and the Zn-ejector solution took place immediately before the 384-well plate was inserted into the spectrophotometer for the first reading. The assay plate was shaken automatically for five seconds after each reading. Readings were taken for 90 cycles at a rate of 20 seconds per cycle, with excitation at 494 nm and emission at 516 nm.

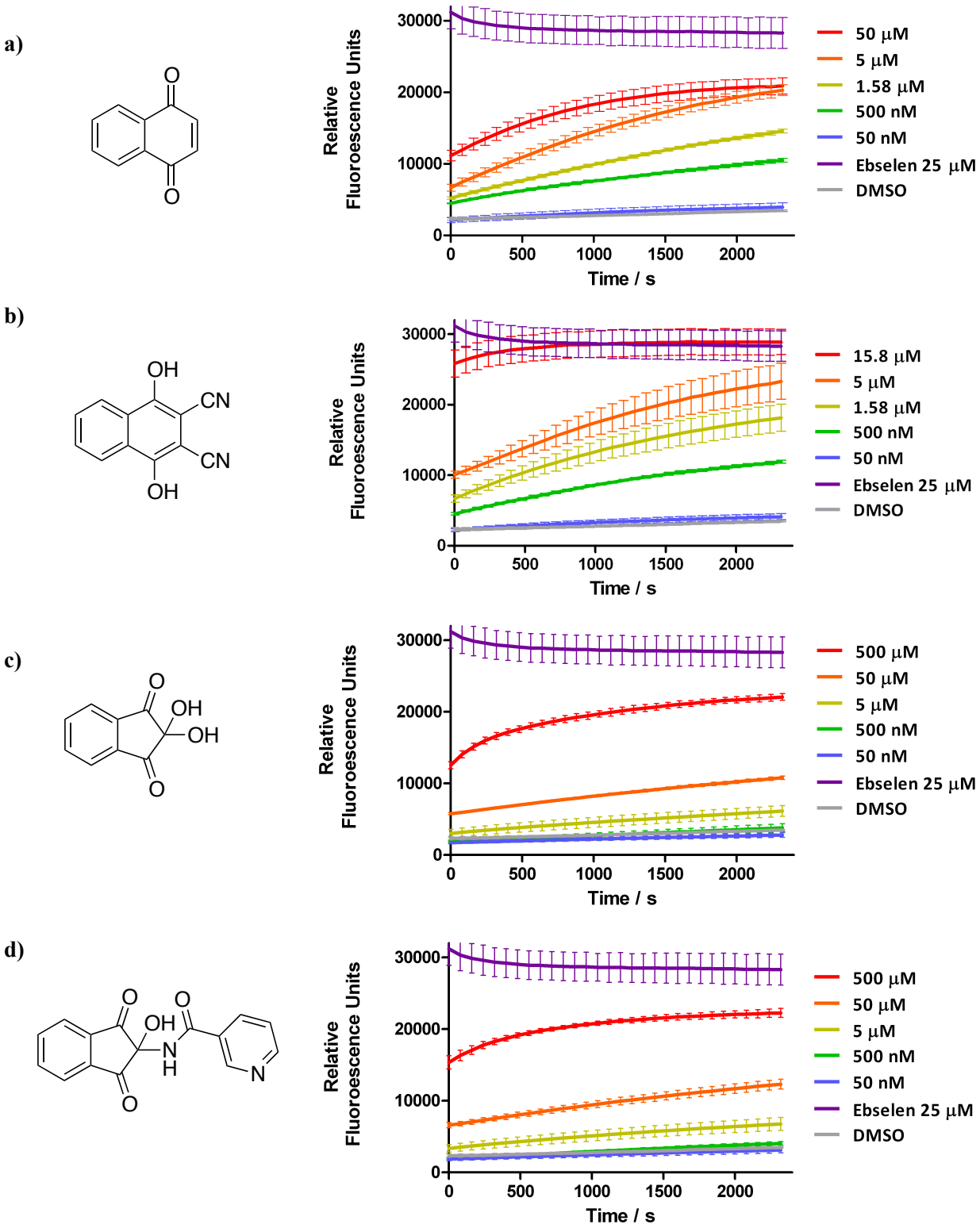


Figure S1. Zinc ejector activity of various compounds against JMJD2A. a) naphthoquinone; b) dihydroxydicyanonaphthene; c) ninhydrin; d) N-(2-hydroxy-1,3-dioxo-2,3-dihydro-1H-inden-2-yl)nicotinamide **6**.

2.3 GST-p300 CH1 FluoZin-3™ Zn ejector Assay

An initial Zn(II) calibration was conducted to ascertain the effect of adding DTT and Tween 0.1 % to the HEPES buffer. All Zn(II) ejector assays with p300 were carried out in a 50 mM HEPES buffer, pH 7.5 with 0.1 % Tween20 and no DTT.

35 µl of GST-p300-$CH1_{323-423}$ (39 µM) was prepared by initially incubating for 4 h with 94.3 µl TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8.0) buffer and 6.8 µl DTT (1 M). 12 µl of 10 µM $ZnCl_2$ was added and the stock was desalted and the buffer exchanged for HEPES (50 mM, pH 7.5, 0.1 % Tween) using a Bio-Rad Micro Bio-Spin 6 chromatography columns. A stock solution of 4.5 µM reduced GST-p300-CH1 in HEPES (50 mM, pH 7.5, 0.1 % Tween) buffer was prepared. 10 µl of 10 µM FZ-3 (50 mM HEPES, pH 7.5, 0.1 % Tween) was mixed with 10 µL of 4.5 µM GST-p300-CH1 solution and 30 µL buffer to make the enzyme mix. A solution of compound in DMSO (5 µL) was mixed with 45 µL buffer. The compound solution was then mixed with the enzyme-FZ-3 solution to give a total volume of 100 µL, with final concentrations of FluoZinTM-3, enzyme and DMSO of 1 µM, 2 µM and 5 %, respectively. Readings were taken as previously described.

Due to excess Zn(II) present in the GST-p300-CH1 stock solution, values were corrected for background and expressed as a percentage of controls (DMSO) to provide the percentage of CH1 binding.

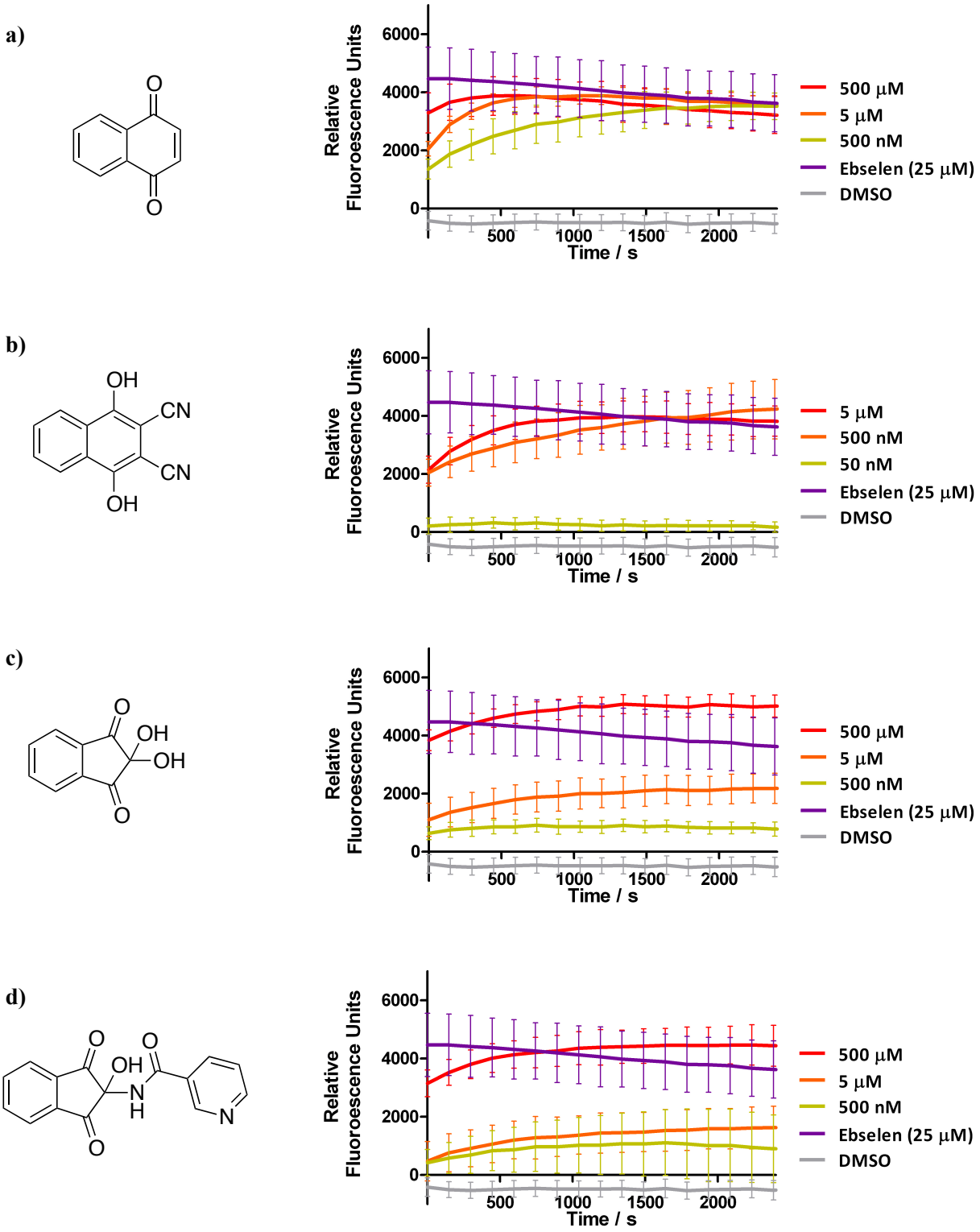


Figure S2. p300 Zinc ejector activity with a) naphthoquinone; b) dihydroxydicyanonaphthene; c) ninhydrin; d) N-(2-hydroxy-1,3-dioxo-2,3-dihydro-1H-inden-2-yl)nicotinamide **6**.

2.4 HIF-1α C-TAD: p300 CH1 Fluorescent Binding Assay

Inhibition of HIF-1α binding to p300 was measured by displacement of GST-p300-$CH1_{323-423}$ from a synthetic biotinylated HIF-1α $C\text{-}TAD_{786-826}$ (Peptide Protein Research Ltd, Fareham, UK) immobilized on 96-well streptavidin-coated plates, as reported by Cook *et al*.[4] GST-p300-CH1 was detected using a europium labelled antibody to GST (Perkin-Elmer). 48.5 nM HIF-1α C-TAD was used to coat plates for 4 h at room temperature. Plates were washed four times with TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8.0) buffer. 7.35 nM GST-CH1 was added with compounds or control (1 % DMSO) in TBST with 5 % BSA, 0.5 mM DTT and 10 μM $ZnCl_2$ and incubated overnight at 4 ˚C. Plates were washed four times with TBST, and europium labelled anti-GST (450 $ng.mL^{-1}$) was added to plates in the buffer used for GST-CH1 addition, and after 2 h, plates were washed six times in TBST. DELFIA enhancement solution (Perkin-Elmer) was added before reading with a Victor3 plate reader (Perkin-Elmer), using the europium setting under time-resolved fluorescence. Values were corrected for background and expressed as a percentage of controls (DMSO) to provide the percentage of CH1 binding. IC_{50} values were calculated using Prism v5.01 (Graphpad) using the nonlinear regression equation log (inhibitor) vs. response-variable slope (Table S1).

Compound	**IC_{50} / μM**
5	1.06 ± 0.03
14	1.99 ± 0.06
15	7.51 ± 0.98
16	0.96 ± 0.01
17	1.27 ± 0.02
23 (ninhydrin)	1.93 ± 0.97

Table S1. IC_{50}'s of amidoindandiones. All compounds displayed similar activity to ninhydrin, their parent compound.

2.5 Cell viability assay

HeLa cells were cultured in DMEM supplemented with 10 % fatal calf serum, 2 mM L-glutamine, 50 units.mL^{-1} of penicillin and 50 µg.mL^{-1} of streptomycin. For cell proliferation assay, HeLa cells were seeded into a 96-well microplate at 1000 cells in 150 µL medium per well. To avoid edge-effects caused by evaporation, only the inner 60 wells of the 96-well microplates were seeded. 24 h after seeding, the cells were treated with inhibitors for a further 24 h and 48 h. Medium was then removed and CyQuant assay was performed using CyQuant NF Cell Proliferation Assay Kit according to manufacturers protocol (Life Technologies). Plates were read using a fluorescence micro plate reader (BMG Labtech FLUOstar Omega) with excitation at 485 nm and emission at 520 nm.

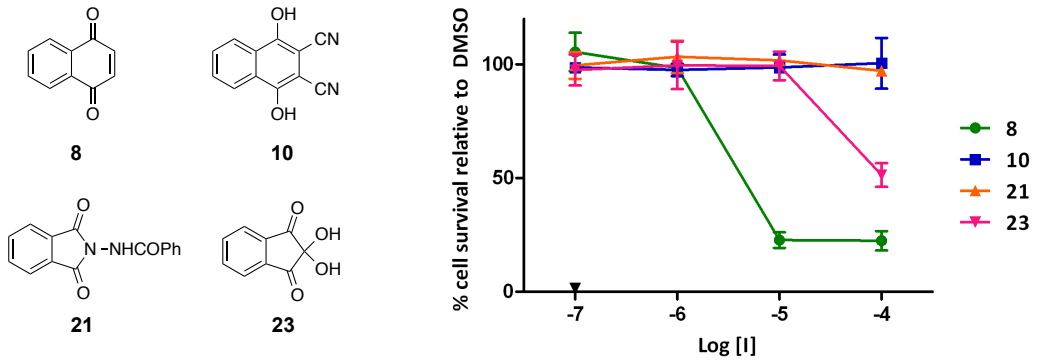


Figure S3. HeLa cell viability studies with HIF-1α/p300 inhibitors. Zn(II) ejectors quinone **8**, reduced quinone **10**, and ninhydrin **21**, and compound **23** (inactive) were analysed for cytotoxicity (48 h), using the Cyquant assay. **10** and **23** do not display cytotoxicity towards Hela cells, whereas **8** and **23** show toxicity at 10 µM and 100 µM respectively . (1% DMSO; triplicate, ±SD)

3. References

[1] A.L. Kung, S.D. Zabludoff, D.S. France, S.J. Freedman, E.A. Tanner, A. Vieira, et al., Small molecule blockade of transcriptional coactivation of the hypoxia-inducible factor pathway, Cancer Cell. 6 (2004) 33–43.

[2] S.S. Ng, K.L. Kavanagh, M.A. McDonough, D. Butler, E.S. Pilka, B.M.R. Lienard, et al., Crystal structures of histone demethylase JMJD2A reveal basis for substrate specificity, Nature. 448 (2007) 87–91.

[3] R. Sekirnik, N.R. Rose, A. Thalhammer, P.T. Seden, J. Mecinović, C.J. Schofield, Inhibition of the histone lysine demethylase JMJD2A by ejection of structural Zn(II), Chem. Commun. (2009) 6376–6378.

[4] K.M. Cook, S.T. Hilton, J. Mecinovic, W.B. Motherwell, W.D. Figg, C.J. Schofield, Epidithiodiketopiperazines block the interaction between hypoxia-inducible factor-1alpha (HIF-1alpha) and p300 by a zinc ejection mechanism, J. Biol. Chem. 284 (2009) 26831–26838.