Electronic Supplementary Information

(ESI)

Fluconazole Analogues with Metal-Binding Motifs Impact Metal-Dependent Processes and Demonstrate Antifungal Activity in *Candida albicans*

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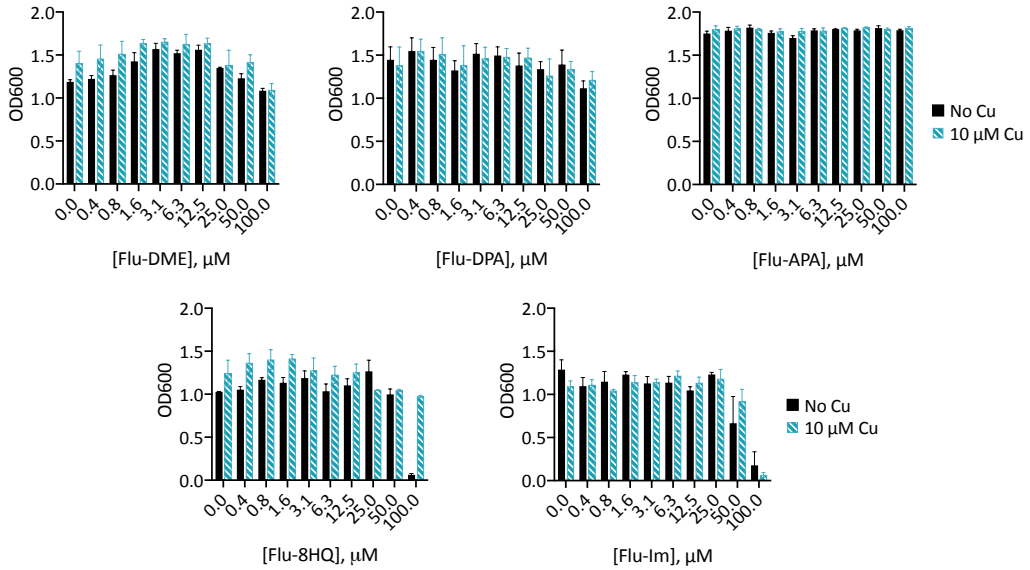

Figure S1. Growth assays with inactive analogues. Flu-DME, Flu-DPA, Flu-APA, Flu-8HQ, and Flu-Im do not fully inhibit growth of *C. albicans* at concentrations below 100 µM, regardless of Cu supplementation. Analogues were tested at concentrations ranging from 0–100 µM with and without 10 µM supplemental $CuSO_4$ added to the medium. Conditions: Growth at 30 °C in YPD (lot 2044606, metal analysis presented in **Table S2, ESI**) monitored by OD600 after 48 h. Data are reported as means with error bars representing standard deviation of three replicate conditions.

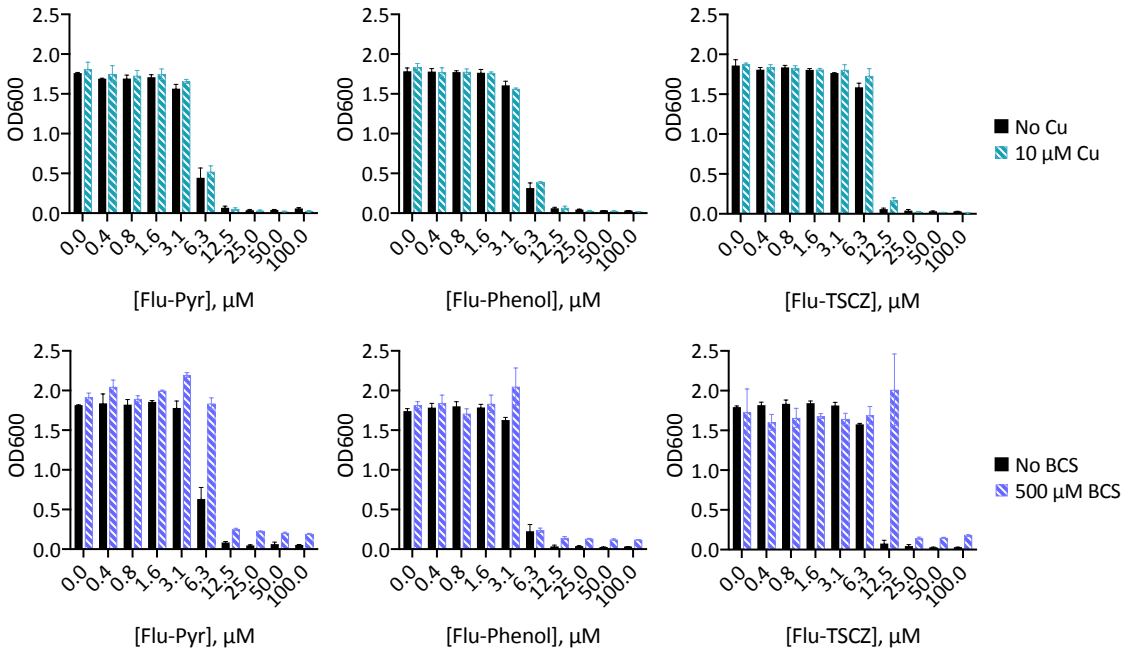

Figure S2. BCS supplementation restores 48-h trailing growth during treatment with Flu-Pyr, Flu-Phenol, and Flu-TSCZ. (top row) Trailing growth was not observed at 48 h for cells treated with Flu-Pyr, Flu-Phenol, and Flu-TSCZ in this particular batch of YPD medium (lot 2044606, metal analysis presented in **Table S2, ESI**). (bottom row) Supplementation with BCS partially rescued growth of analogue-treated cells. Conditions: Growth at 30 °C in YPD monitored at OD600 after 48 h. Data are reported as means with error bars representing standard deviation of three replicate conditions.

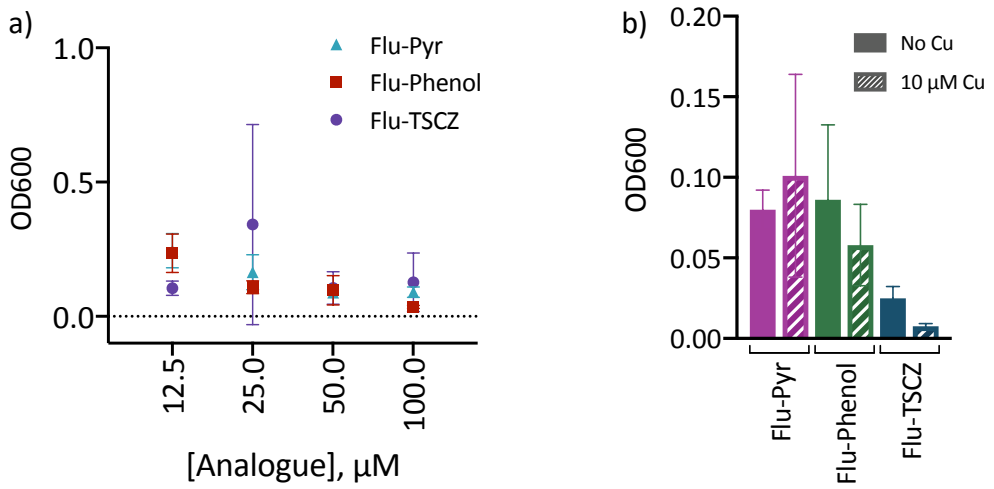

Figure S3. Trailing growth in YPD medium. (a) Trailing growth is evident following 72 h of treatment with Flu-Pyr, Flu-Phenol, and Flu-TSCZ. Conditions: Growth at 30 °C in YPD medium (lot 2044606) monitored at OD600 after 72 h. (b) 48-h growth of cells treated with 100 µM Flu-Pyr, Flu-Phenol, or Flu-TSCZ +/- 10 µM $CuSO_4$ in YPD medium prior to CFU determination. Data are reported as means with error bars representing standard deviation of three replicate conditions.

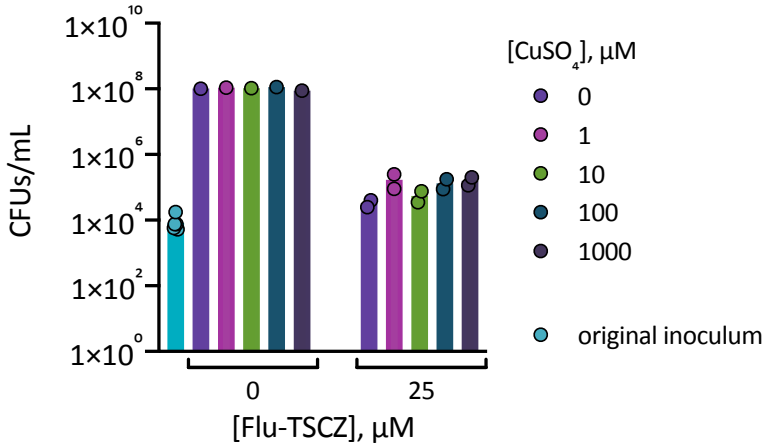

Figure S4. Colony forming units (CFUs) following 48 h of exposure to 0 or 25 µM Flu-TSCZ. Cultures treated with 25 µM Flu-TSCZ had fewer CFUs/mL than cultures treated with 0 µM Flu-TSCZ, demonstrating inhibitory activity, but slightly higher CFUs/mL than the original inoculum, indicating the presence of trailing growth. Varying the concentration of Cu in the growth medium did not affect CFUs/mL of cultures exposed to 0 or 25 µM Flu-TSCZ. Conditions: Growth at 30 °C in liquid YPD (lot 2044606, metal analysis presented in **Table S2, ESI**) for 48 h, then spotted on YPD agar and colonies counted after 24 h incubation at 30 °C. Data are reported as means with individual data points indicating replicates within a single experiment.

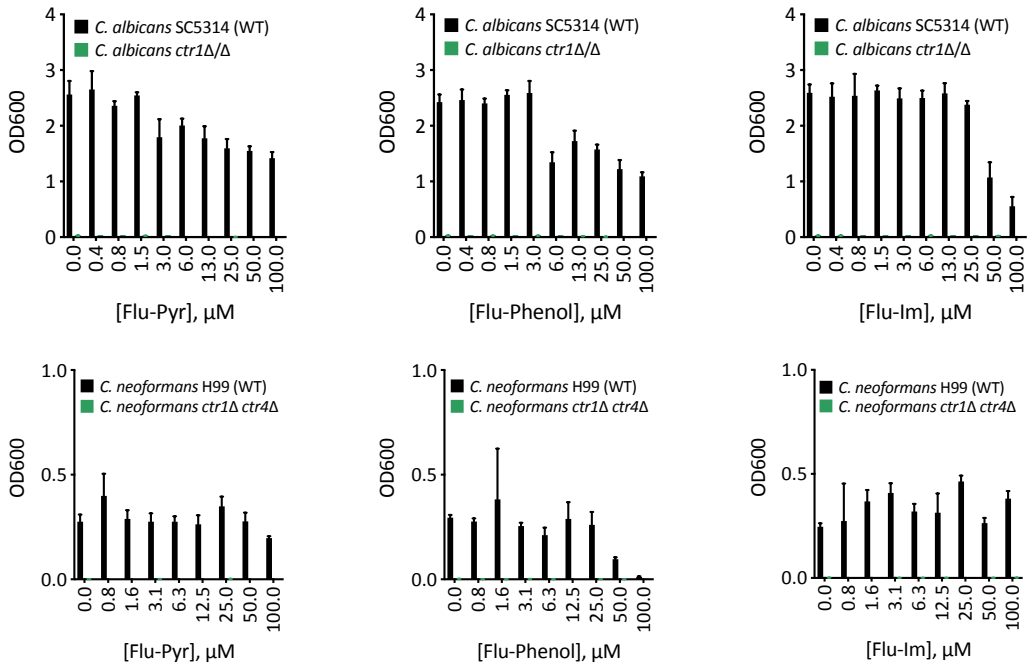

Figure S5. Flu-Pyr, Flu-Phenol, and Flu-Im do not recover growth of cells lacking Cu import machinery. 48 h-growth of *C. albicans* (top) or *C. neoformans* (bottom) WT (black bars) and Cu import mutants (green bars) treated with 0–100 µM analogue in YPEG medium. No growth was observed for cells lacking Cu import genes under these conditions, even with analogue treatment. Data are reported as means with error bars representing standard deviation of three replicate conditions.

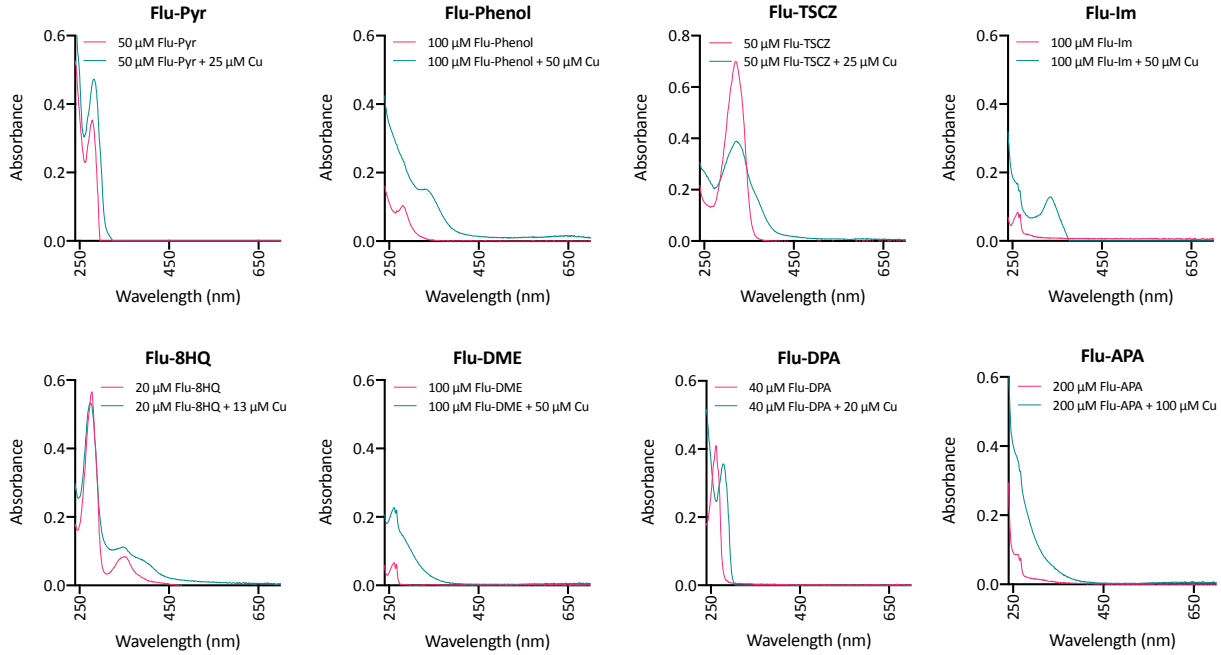

Figure S6. All eight analogues bind Cu(II) in HEPES buffer. UV-Vis spectra of analogues and their Cu(II) complexes in 50 mM HEPES buffer, pH 7.4. Analogue and $CuSO_4$ concentrations are indicated in figure legends.

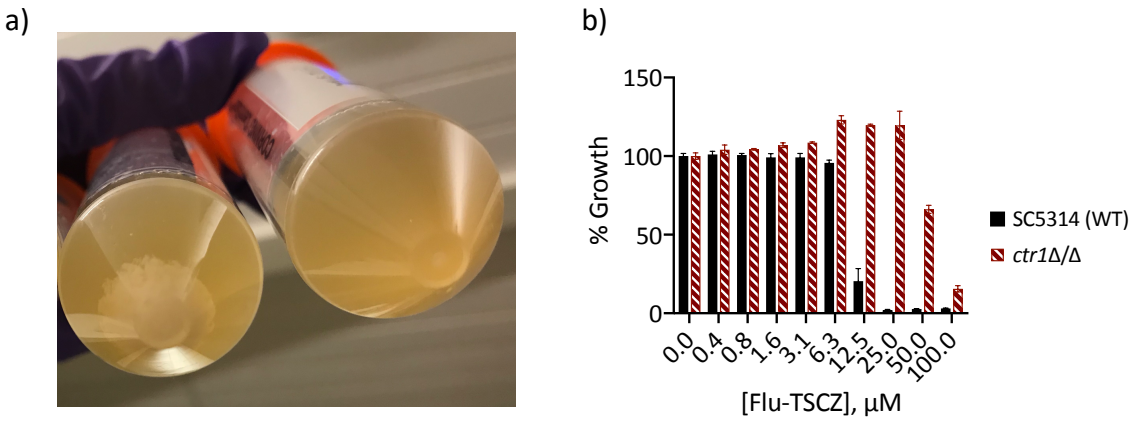

Figure S7. *C. albicans ctr1*Δ/Δ strain exhibits reduced susceptibility to Flu-TSCZ, regardless of culture morphology. (a) Side-by-side comparison of *ctr1*Δ/Δ cultures after overnight growth, each prepared by inoculating YPD medium with a single colony of the *ctr1*Δ/Δ strain. The culture on the right contained only planktonic cells, but in the culture on the left, cells grew in clumps. (b) The clumped cells were treated with Flu-TSCZ in YPD medium at 30 °C for 48 h then growth was determined by measuring OD600. Data are normalized to the untreated control for each strain and reported as means with error bars representing standard deviation of three replicate conditions.

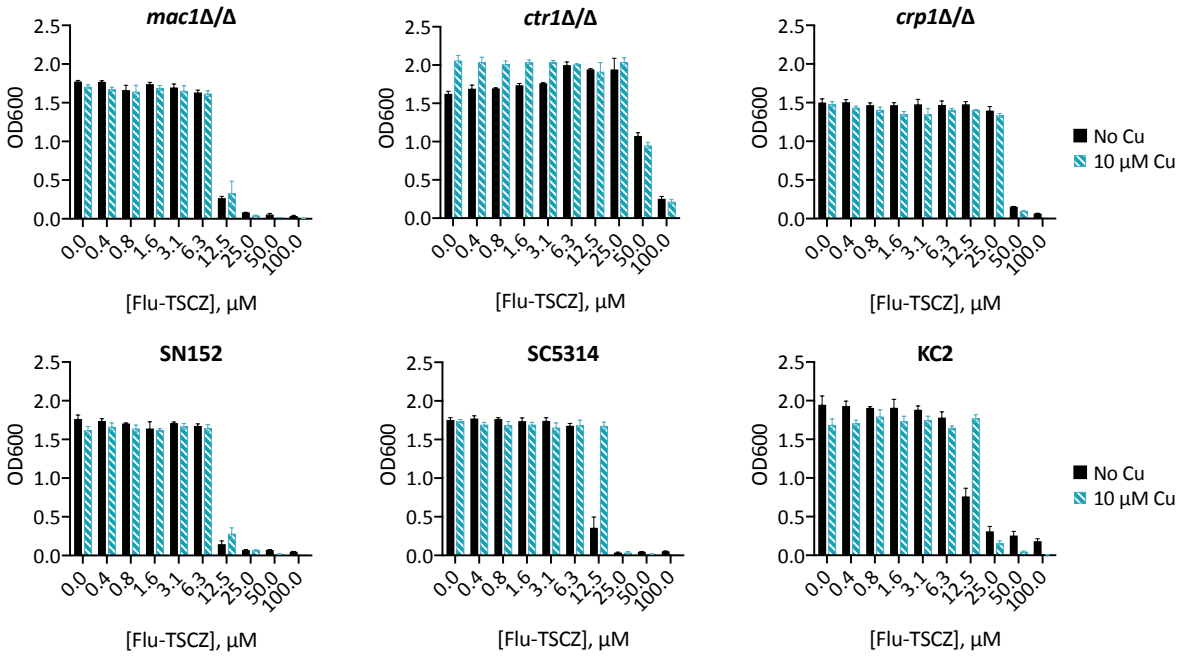

Figure S8. Effect of Cu supplementation on growth of *C. albicans mac1*Δ/Δ, *ctr1*Δ/Δ, and *crp1*Δ/Δ cells and their isogenic parent strains SN152, SC5314, and KC2, respectively, during treatment with Flu-TSCZ. In the deletion strains, Cu supplementation did not impact growth inhibition by Flu-TSCZ, though it did slightly improve overall growth of the *ctr1*Δ/Δ strain. In the parent strains, Cu supplementation had some impact, most clearly the growth rescue observed in SC5314 at one concentration of Flu-TSCZ.

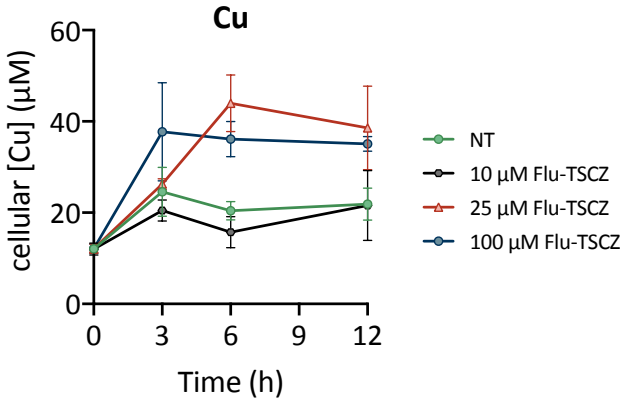

Figure S9. Comparison of cellular Cu content (µM) following treatment with different concentrations of Flu-TSCZ. Cell-associated Cu levels were approximately doubled following 6 h of treatment with 25 or 100 µM Flu-TSCZ, relative to levels in untreated cells or cells treated with only 10 µM Flu-TSCZ. Cell-associated Cu levels were analyzed by ICP-MS and cellular Cu concentrations were calculated as described in Methods. Data are reported as mean ± SEM, *n* = 3 biologically independent samples per timepoint.

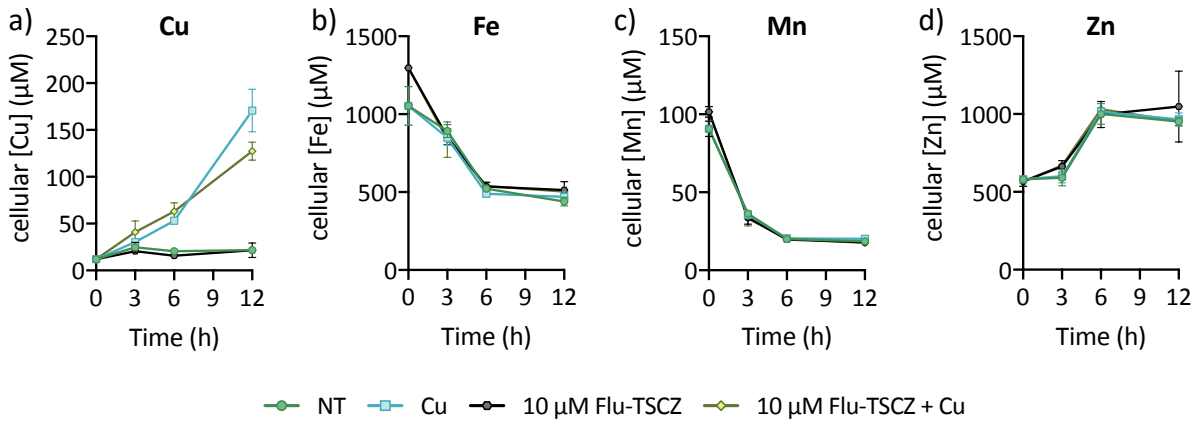

Figure S10. Metal content of *C. albicans* cells reported as cellular concentration (µM) following treatment with 10 µM Flu-TSCZ. Treatment with 10 µM Flu-TSCZ (black hexagon) was not sufficient to induce changes in levels of Cu (a), Fe (b), Mn (c), or Zn (d) relative to untreated cells (green circles). Supplementation with Cu (blue squares) increased cell-associated Cu over that of untreated but treating Cu-supplemented cells with 10 µM Flu-TSCZ (lime green diamonds) had no additional effect. Cell-associated metal levels were analyzed by ICP-MS and cellular metal concentrations were calculated as described in Methods. Data are reported as mean ± SEM, *n* = 3 biologically independent samples per timepoint.

Table S1: Metal content of YPD medium (µM) lot #2005064

Trial	Mg	Ca	Cu	Fe	Mn	Zn	Co	Ni
Conc ± CoV	210 ± 20	131 ± 2	0.143 ± 0.004	11.2 ± 0.2	0.166 ± 0.003	22.1 ± 0.4	0.213 ± 0.004	0.143 ± 0.003

Table S2: Metal content of YPD medium (µM) lot #2044606

Trial	Mg	Ca	Cu	Fe	Mn	Zn	Co	Ni
Conc ± CoV	130 ± 10	100 ± 2	0.165 ± 0.005	8.7 ± 0.1	0.303 ± 0.005	17.4 ± 0.3	0.34 ± 0.01	0.080 ± 0.002

Table S3: Metal content of YPD medium (µM) lot #2101669

	Mg	Ca	Cu	Fe	Mn	Zn	Co	Ni
Conc ± CoV	119 ± 2	108 ± 2	0.22 ± 0.01	2.57 ± 0.06	0.288 ± 0.004	19.2 ± 0.3	0.31 ± 0.01	0.087 ± 0.002

Table S4: Metal content of Tris:SD Cu Drop-Out medium (µM)

Trial	Mg	Ca	Cu	Fe	Mn	Zn	Co	Ni
Conc ± CoV	248 ± 4	560 ± 9	0.065 ± 0.002	0.057 ± 0.001	0.132 ± 0.002	0.288 ± 0.005	0.00175 ± 0.00004	0.0156 ± 0.0003

Table S5: Metal content of Tris:SD Fe Drop-Out medium (µM)

Trial	Mg	Ca	Cu	Fe	Mn	Zn	Co	Ni
Conc ± CoV	243 ± 4	548 ± 9	0.184 ± 0.004	0.046 ± 0.001	0.213 ± 0.003	0.34 ± 0.01	0.00187 ± 0.00004	0.0171 ± 0.0004

Table S6: Metal content of Tris:SD Mn Drop-Out medium (µM)

Trial	Mg	Ca	Cu	Fe	Mn	Zn	Co	Ni
Conc ± CoV	234 ± 4	540 ± 8	0.193 ± 0.005	0.081 ± 0.002	0.0037 ± 0.0001	0.34 ± 0.01	0.00158 ± 0.00003	0.0174 ± 0.0004

Table S7: Metal content of Tris:SD Zn Drop-Out medium (µM)

Trial	Mg	Ca	Cu	Fe	Mn	Zn	Co	Ni
Conc ± CoV	263 ± 5	546 ± 9	0.179 ± 0.004	0.062 ± 0.001	0.238 ± 0.003	0.088 ± 0.002	0.00153 ± 0.00003	0.0158 ± 0.0003

Table S8: List of Strains Used in this Study

Name	Yeast	Genotype	Source/Description
SC5314	*C. albicans*	Wild-type	Obtained from the American Type Culture Collection. Wild-type strain used in the *C. albicans* sequencing project [1].
*ctr1*Δ/Δ	*C. albicans*	*ctr1Δ::loxP/ctr1Δ::loxP*	Obtained from the Brown lab at the University of Exeter. Originally reported by Mackie et al [2].
SN152	*C. albicans*	*his1Δ/his1Δ*, *leu2Δ/leu2Δ*, *arg4Δ/arg4Δ*, *URA3/ura3Δ*::*imm434*, *IRO1/iro1Δ*::*imm434*	Obtained from the Fungal Genetics Stock Center [3]. Originally reported by Noble et al [4].
*mac1*Δ/Δ	*C. albicans*	*mac1Δ*::*LEU2/mac1Δ*::*HIS1*	Obtained from the Fungal Genetics Stock Center [3]. Originally reported by Homann et al [5].
KC2	*C. albicans*	*ura3Δ*::*imm434/ura3Δ*::*imm434*	Obtained from the Culotta lab at Johns Hopkins University. Also called CAF3-1. Originally reported by Fonzi and Irwin [6].
*crp1*Δ/Δ	*C. albicans*	*crp1Δ*::*hisG/crp1Δ::hisG*	Obtained from the Culotta lab at Johns Hopkins University. Originally reported by Weissman et al [7].
H99	*C. neoformans*	Wild-type	Obtained from the Thiele lab at Duke University.
*ctr1*Δ *ctr4*Δ	*C. neoformans*	*ctr1::NAT; ctr4::Neo*	Obtained from the Thiele lab at Duke University. Originally reported by Ding et al [8].

NMR Spectra

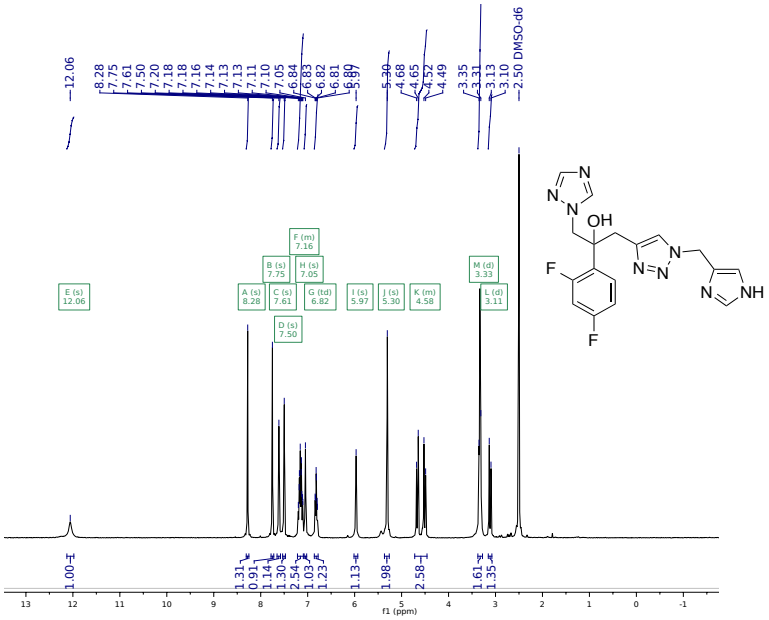

Figure S12: ^{1}H NMR spectrum of Flu-Im (I-a) in DMSO.

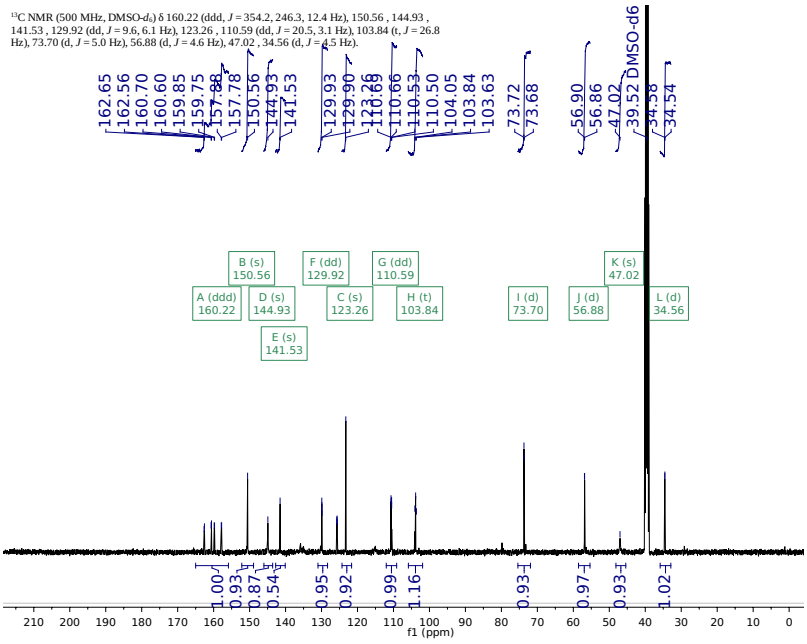

Figure S13: ^{13}C NMR spectrum of Flu-Im (I-a) in DMSO.

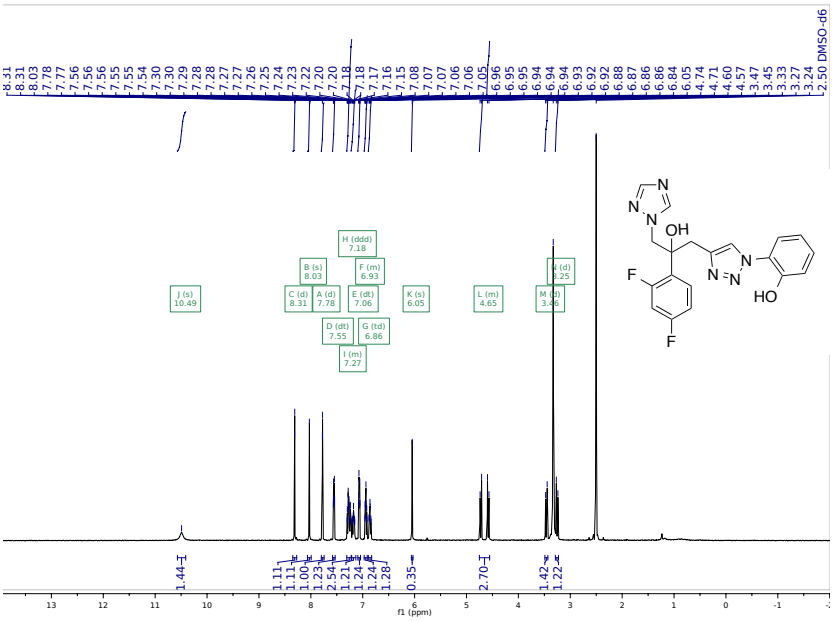

Figure S14: ^{1}H NMR spectrum of Flu-Phenol (I-b) in DMSO.

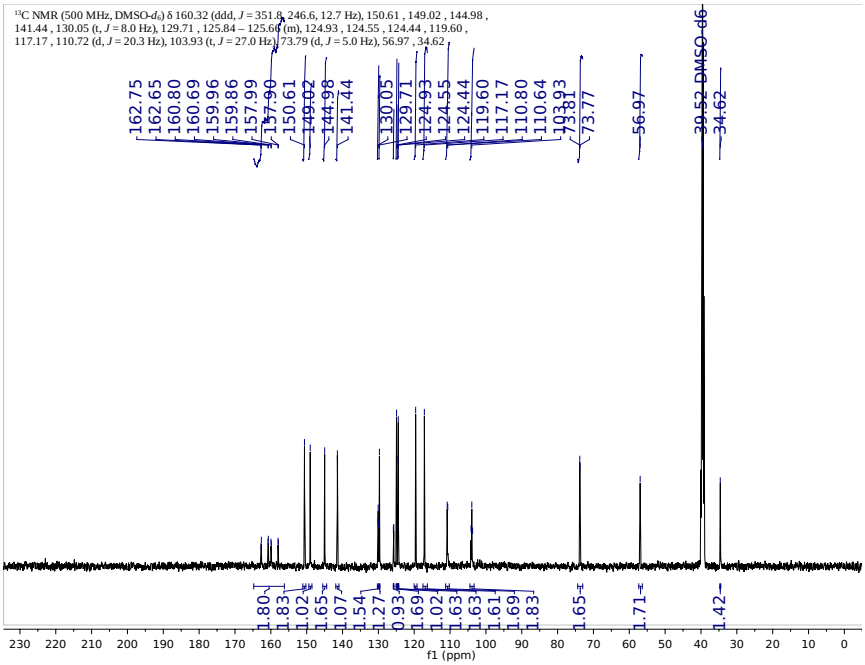

Figure S15: ^{13}C NMR spectrum of Flu-Phenol (I-b) in DMSO.

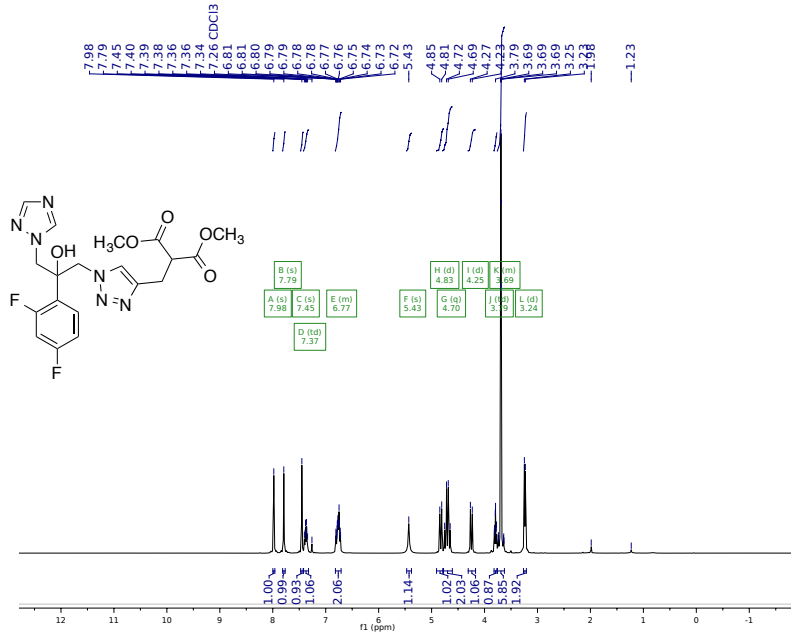

Figure S16: ^{1}H NMR spectrum of Flu-DME (II-a) in $CDCl_3$.

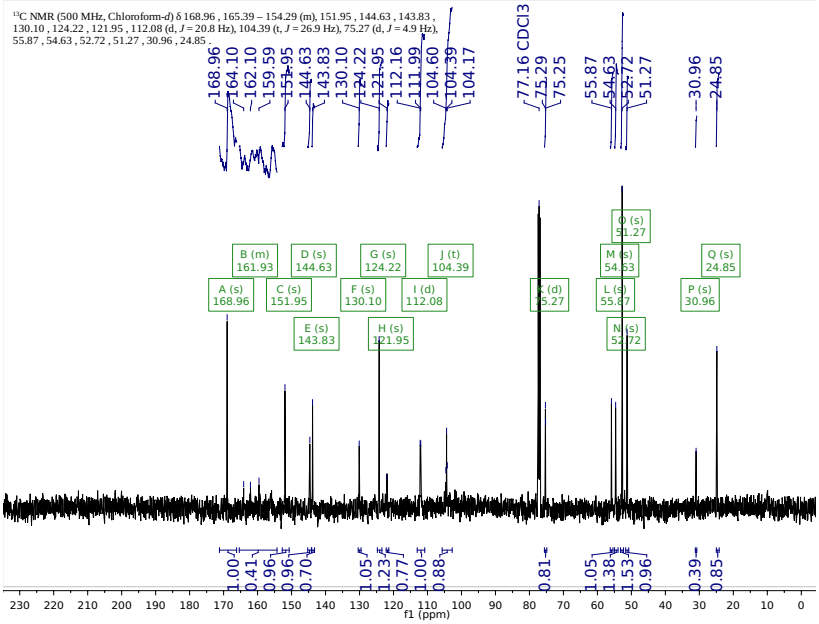

Figure S17: ^{13}C NMR spectrum of Flu-DME (II-a) in $CDCl_3$.

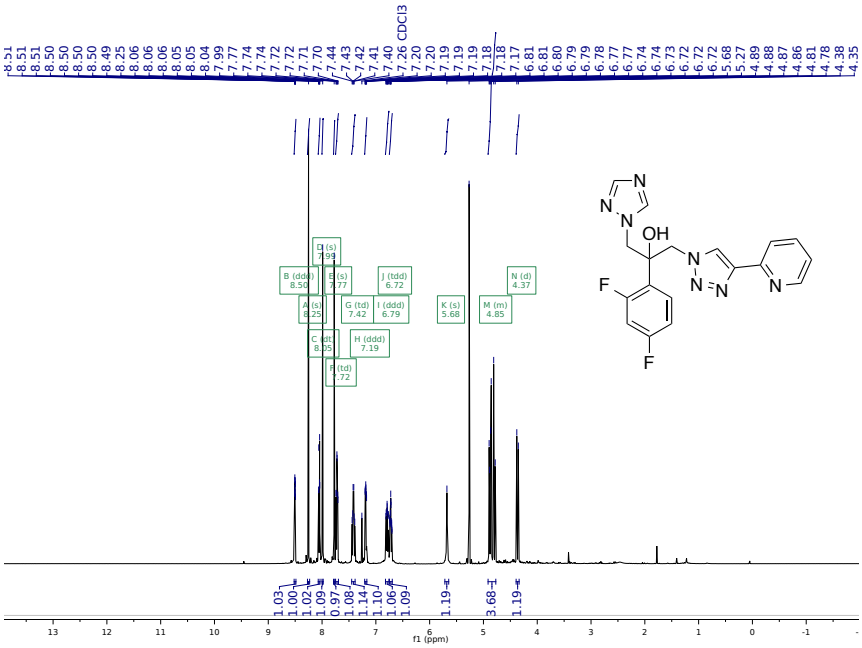

Figure S18: ^{1}H NMR spectrum of Flu-Pyr (II-b) in $CDCl_3$.

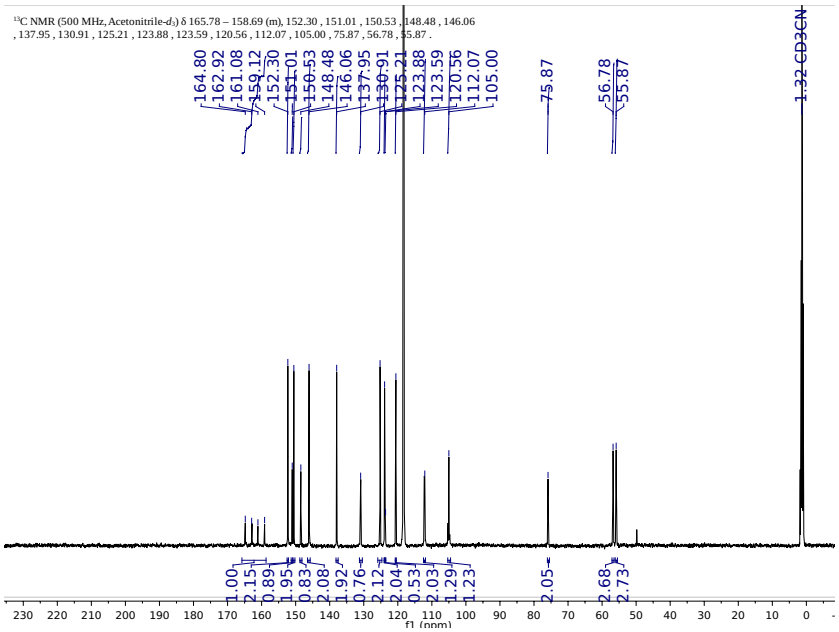

Figure S19: ^{13}C NMR spectrum of Flu-Pyr (II-b) in CD_3CN.

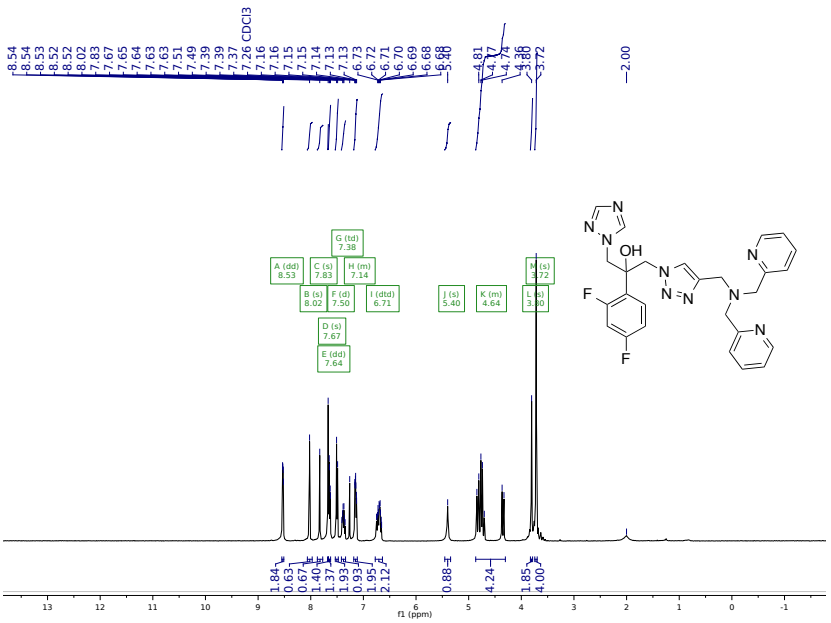

Figure S20: ^{1}H NMR spectrum of Flu-DPA (II-c) in $CDCl_3$.

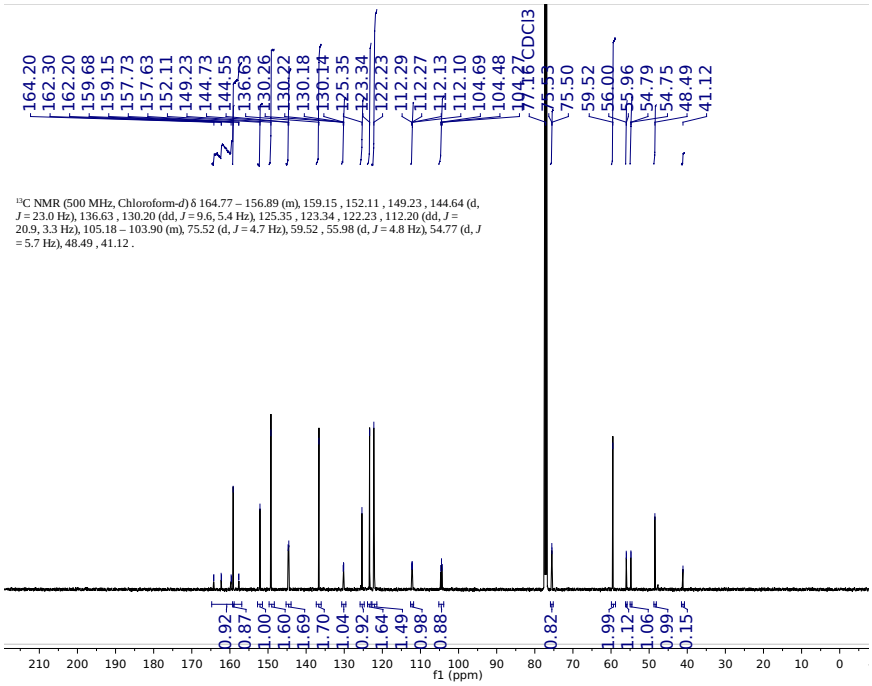

Figure S21: ^{13}C NMR spectrum of Flu-DPA (II-c) in $CDCl_3$.

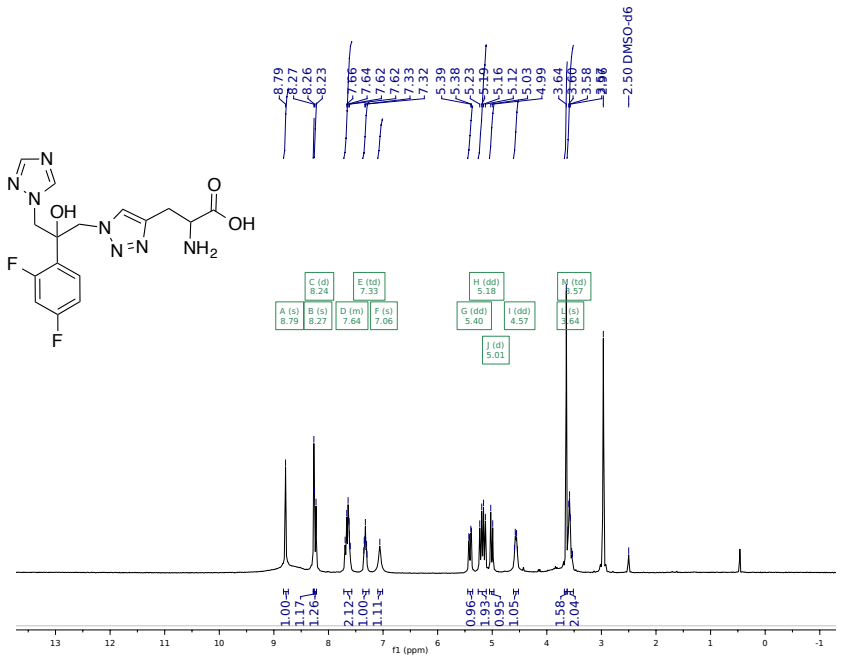

Figure S22: ^{1}H NMR spectrum of Flu-APA (II-d) in DMSO.

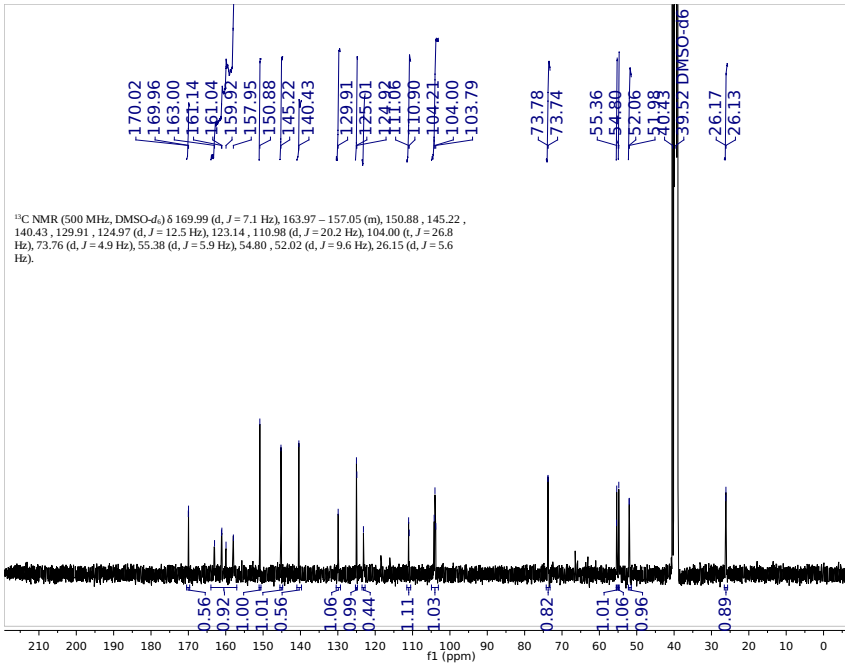

Figure S23: ^{13}C NMR spectrum of Flu-APA (II-d) in DMSO.

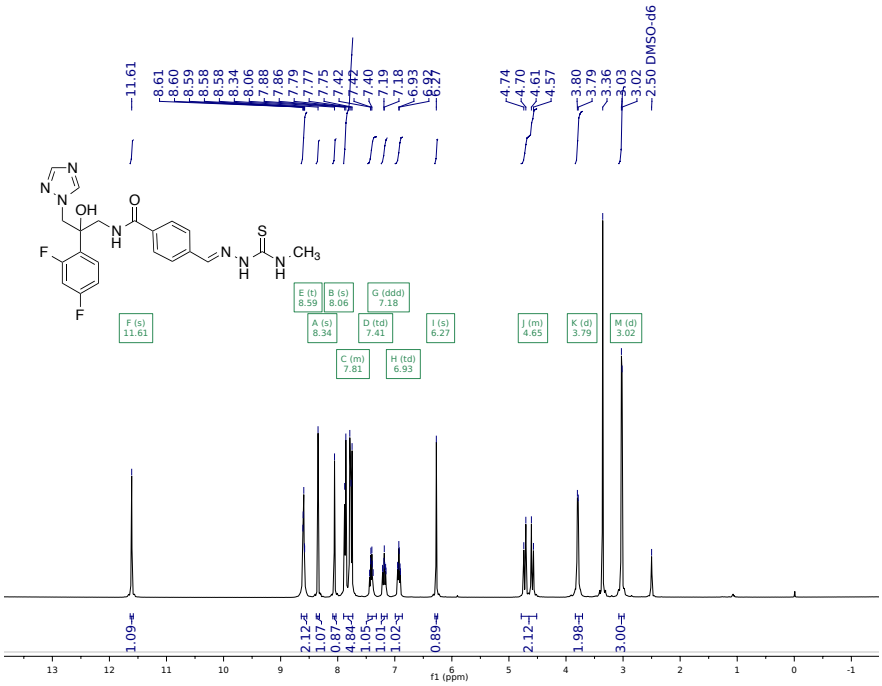

Figure S24: ^{1}H NMR spectrum of Flu-TSCZ (III-a) in DMSO.

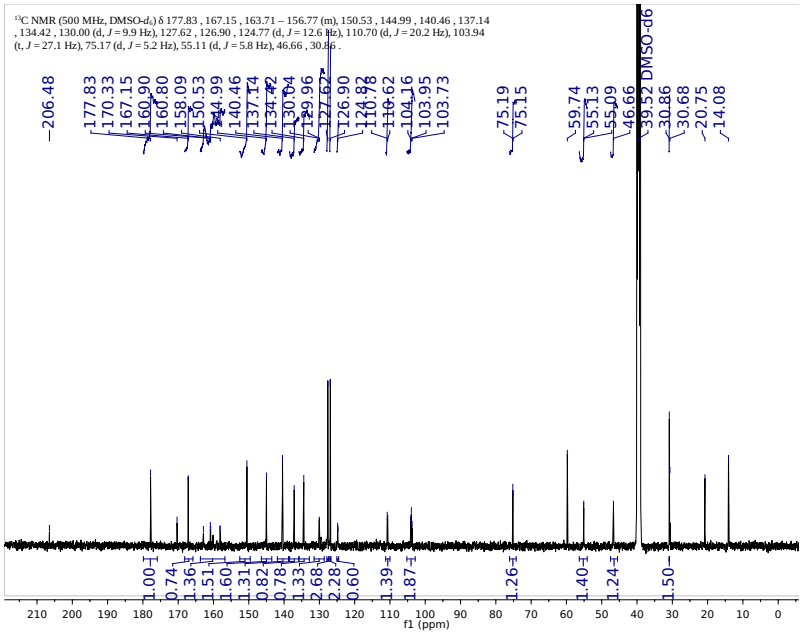

Figure S25: ^{13}C NMR spectrum of Flu-TSCZ (III-a) in DMSO.

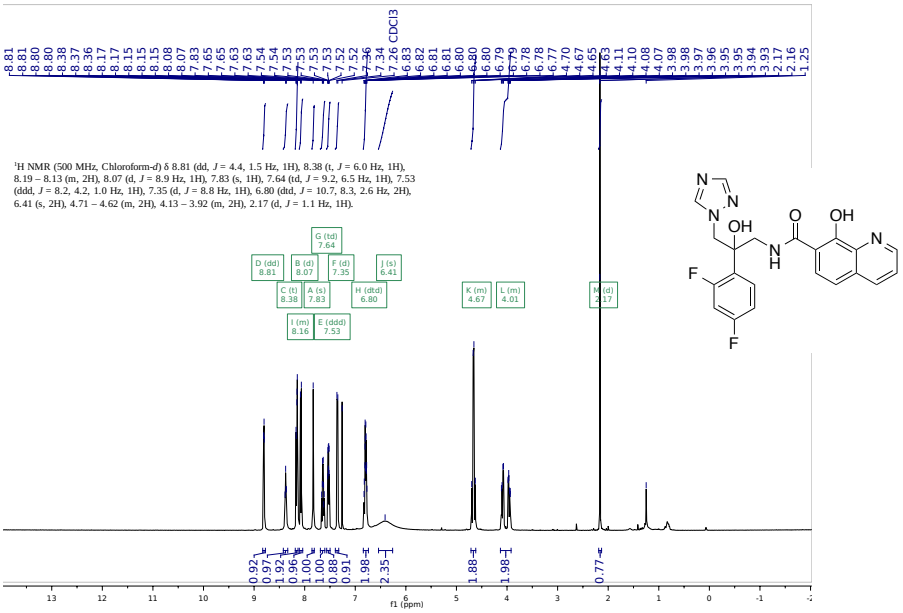

Figure S26: ^{1}H NMR spectrum of Flu-8HQ (III-b) in $CDCl_3$.

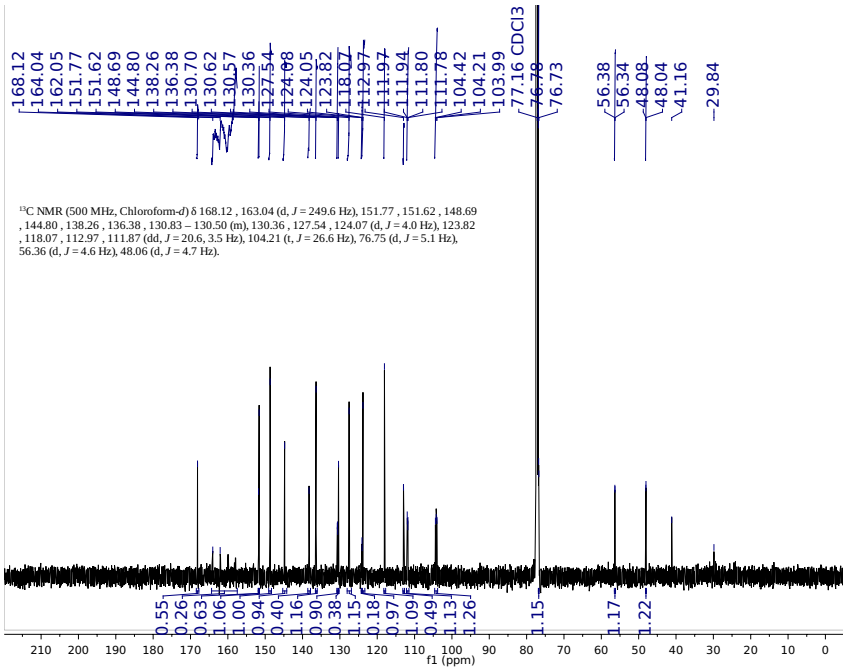

Figure S27: ^{13}C NMR spectrum of Flu-8HQ (III-b) in $CDCl_3$.

Supplementary References

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