Synthesis, characterization, and bioactivity of the photoisomerizable tubulin polymerization inhibitor azo-combretastatin A4

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CONTENTS:

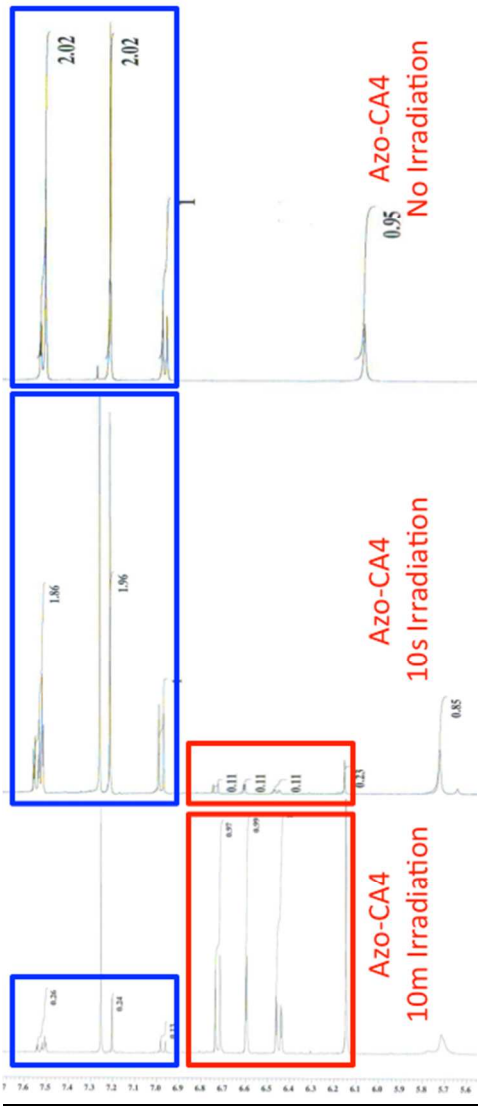


Figure S1. ^{1}H HMR analysis of azo-CA4 in the dark (top), with 10 s irradiation (middle), and 10 min irradiation (bottom).

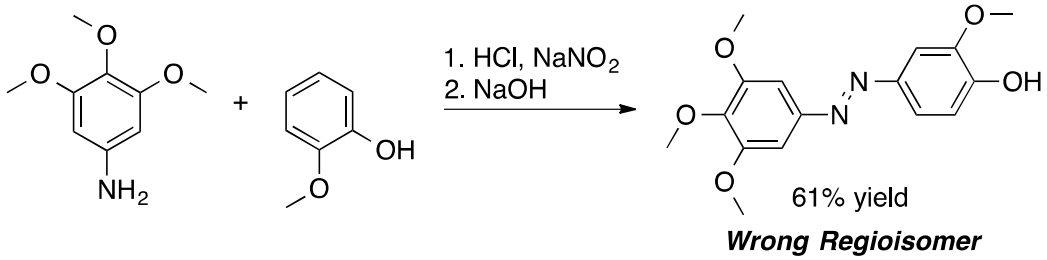
Azo-CA4 (10 mM in $CDCl_3$) was irradiated on a watch glass with a 400 nm LED for the times specified. The sample was then immediately subjected to NMR analysis. With this wavelength, the cis:trans ratio has a maximum of ~9:1 in under 10 minutes. However, lower concentrations of azo-CA4 required much shorter irradiation times to achieve maximum isomerization.

General Synthetic Experimental Information:

Reagents and solvents were purchased from Alfa Aesar, Acros Organics, Fisher Scientific, Pharmco-Aaper, and Sigma Aldrich. All NMR solvents were purchased from Cambridge Isotope Laboratories. NMR spectra were collected with a JEOL Eclipse +400 FT NMR Spectrometer. IR data was collected with a Jasco FT/IR-4200.

Single-Step Azo-Coupling of 3,4,5-trimethoxyaniline and 2-methylanisole

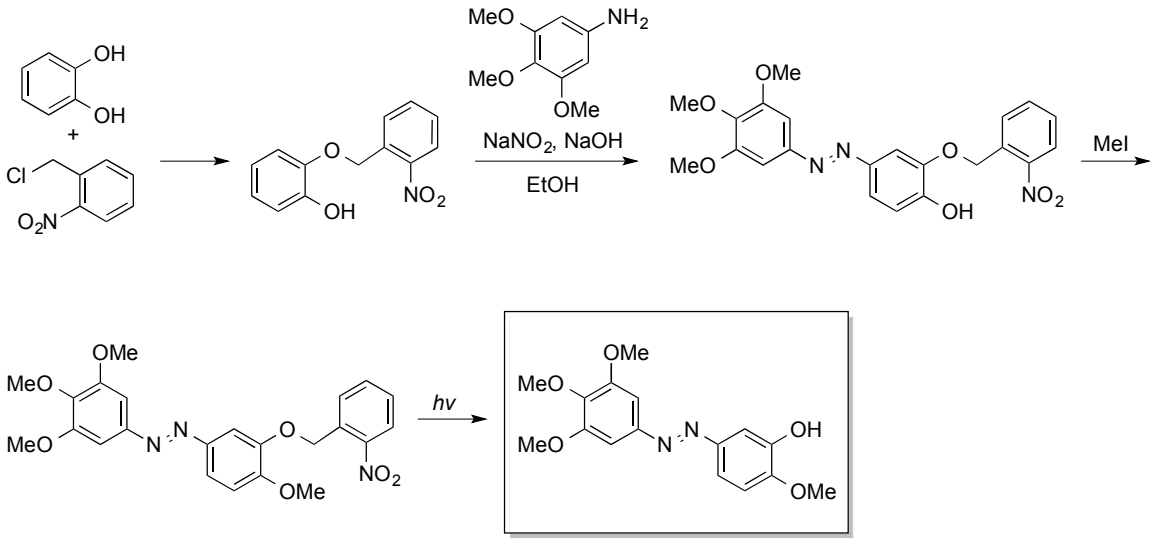
Astonishingly, despite the vast array of azo-stilbenoids that have been previously synthesized, at the outset of this project, azo-CA4 had not been previously reported.‡ A brief review of the literature suggests that in the vast majority of all previously synthesized azo-stilbenes, the azo and phenol functional groups are para. This is an artifact of the mechanism of the single step sodium nitrite mechanism, which produces this regioisomer. As a result, azo-stilbenes with other structures are largely unexplored. Still, given the donating nature of both substituents in 2-methylanisole, we attempted to synthesize azo-CA4 in a single step, understanding that the desired regioisomer would be least favorable. However, in our hands, we could only isolate the undesired regioisomer, necessitating a longer synthesis utilizing protecting groups.



Scheme S1. Single step azo coupling between 3,4,5-trimethoxyaniline and 2-methylanisole produces the undesired azo-stilbenoid regioisomer.

First Generation Synthesis of Azo-Combretastatin A4

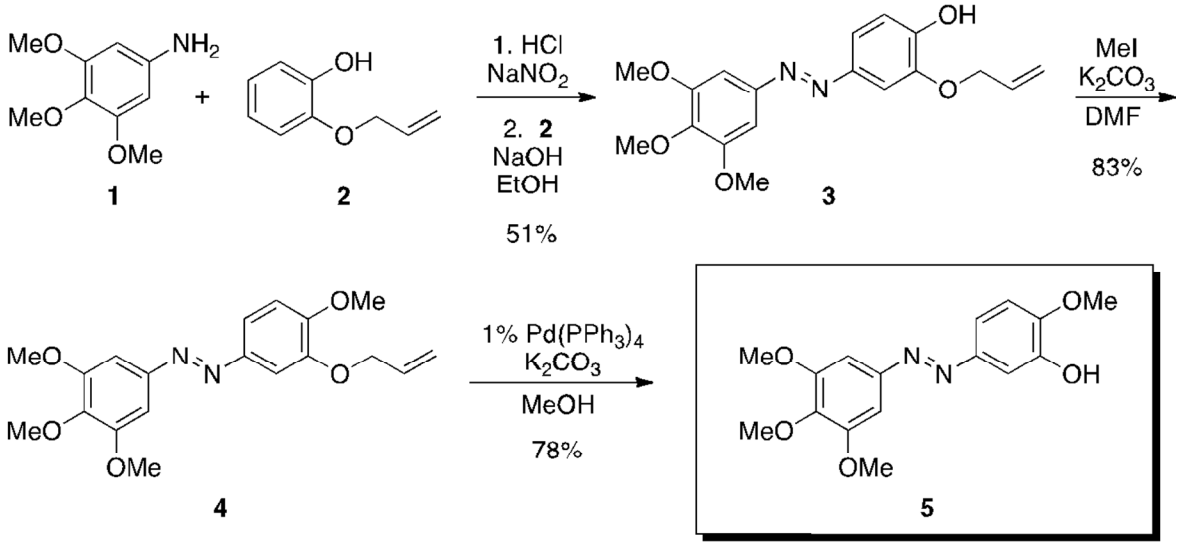
Given the relatively harsh acidic and basic conditions necessary for azo-bond formation, we began with the robust ortho-nitrobenzyl (ONB) protecting group that can be orthogonally removed with light. While small quantities (~1%) of azo-CA4 were isolated via this route, the strong light source necessary to cleave the ONB protecting group also obliterated the azo-CA4, necessitating a search for another synthetic route.



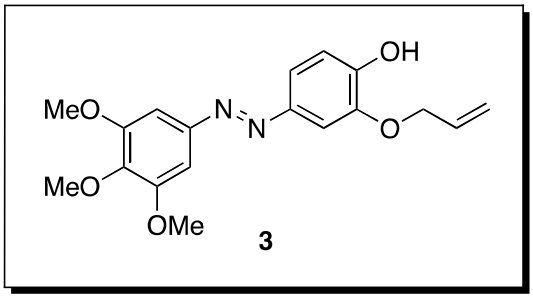
Scheme S2. First generation synthesis of azo-CA4.

Second Generation Synthesis of Azo-Combretastatin A4

Given the relatively harsh acidic and basic conditions necessary for azo-bond formation, and the difficulty photolysing ONB, we designed a second-generation synthesis using allyl-protected catechol, which was orthogonally removed with a palladium catalyst. This synthesis produced large quantities of azo-CA4 (5).



Scheme S3. Second-generation synthesis of azo-CA4.



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Compound **3**. 3,4,5-trimethoxyaniline (0.66 g, 3.6 mmol) was dissolved in approximately 20 mL ethanol and chilled to -10 °C. Then 3 M HCl (3.0 mL, 9.0 mmol) was added dropwise via syringe, followed by dropwise addition of 2.5 M $NaNO_2$ (1.4 mL, 3.6 mmol) resulting in a color change to deep red. The reaction was left to stir at -15 °C for approximately 45 minutes before dropwise addition of compound **2** (0.60 g, 4.0 mmol) dissolved in 2 M NaOH (3.6 mL, 7.2 mmol) causing the reaction to darken. The flask was allowed to warm to room temperature, and left to stir for two hours. The reaction was then quenched with 3 M HCl (3.0 mL, 9 mmol) and extracted twice into CH_2Cl_2 (2x50 mL). The combined organic layers were washed with saturated $NaHCO_3$, dried with Na_2SO_4, and concentrated onto diatomaceous earth. The desired product was isolated by automated flash chromatography with hexane:ethyl acetate mobile phase (0->10% EtOAc gradient over 20 minutes) to produce a light orange solid in (636 mg) 51% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm) 7.58 (dd, J = 8.4, 2.2 Hz, 1H), 7.48 (d, J = 1.8 Hz, 1H), 7.20 (s, 2H), 7.06 (d, J = 8.4 Hz, 1H), 6.11 (ddt, J = 17.2, 10.6, 5.4 Hz, 1H), 6.00 (s, 1H), 5.47 (ddd, J = 17.4, 2.8, 1.5 Hz, 1H), 5.36 (ddd, J = 10.4, 2.6, 1.1 Hz, 1H), 4.71 (dt, J = 5.3, 1.2 Hz, 2H), 3.96 (s, 6H), 3.92 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ(ppm) 153.6, 148.9, 148.6, 146.5, 146.1, 140.1, 132.4, 121.2, 119.0, 114.5, 103.3, 100.1, 70.0, 61.1, 56.3; IR (thin film) cm^{-1} 3404, 3081, 2999, 2934, 2837, 1595, 1497, 1466, 1422, 1408, 1329, 1311, 1268, 1232, 1178, 1128, 1006, 927, 858, 819, 797, 733, 654.

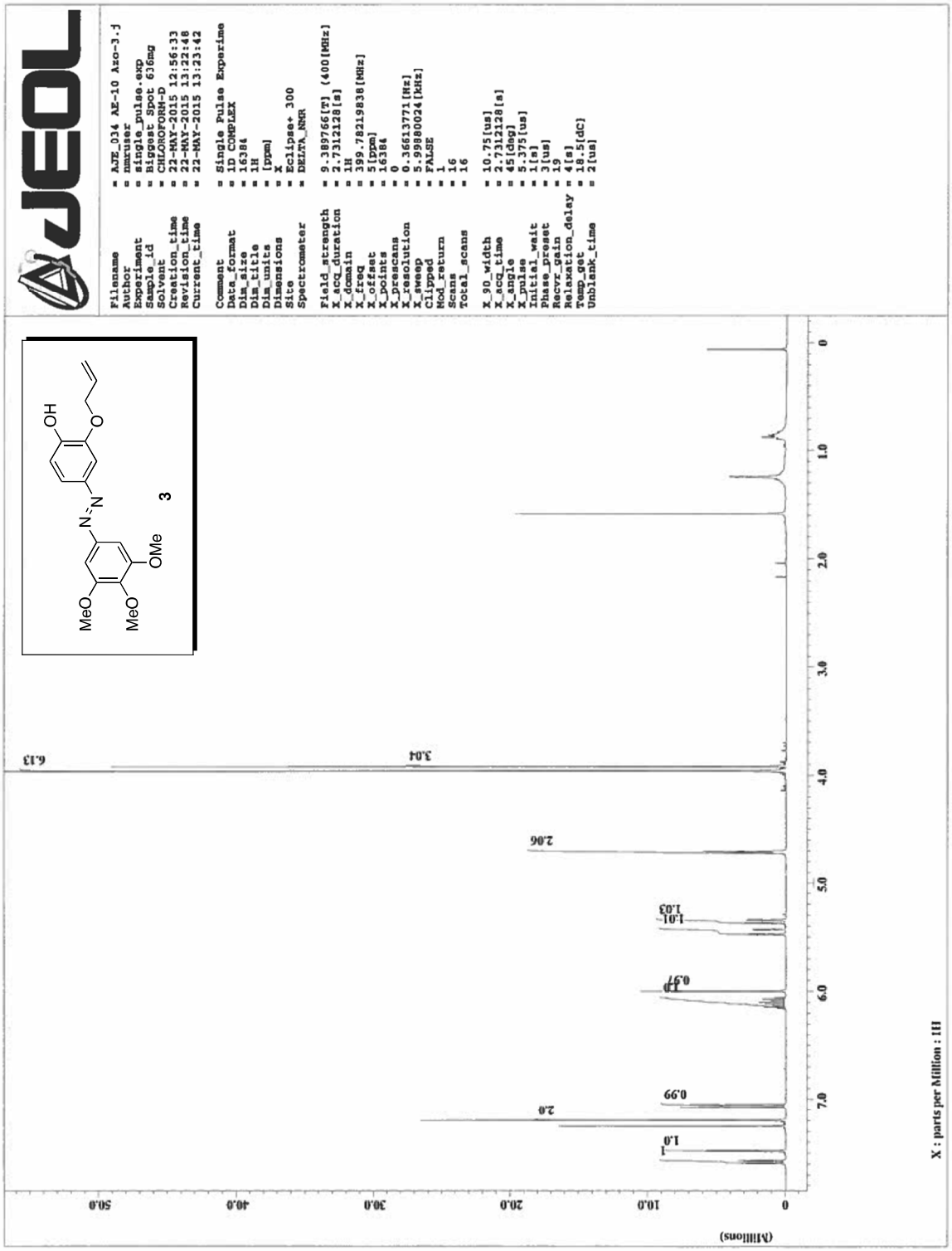


Figure S2. ^{1}H NMR of Compound 3 (400 MHz, $CDCl_3$).

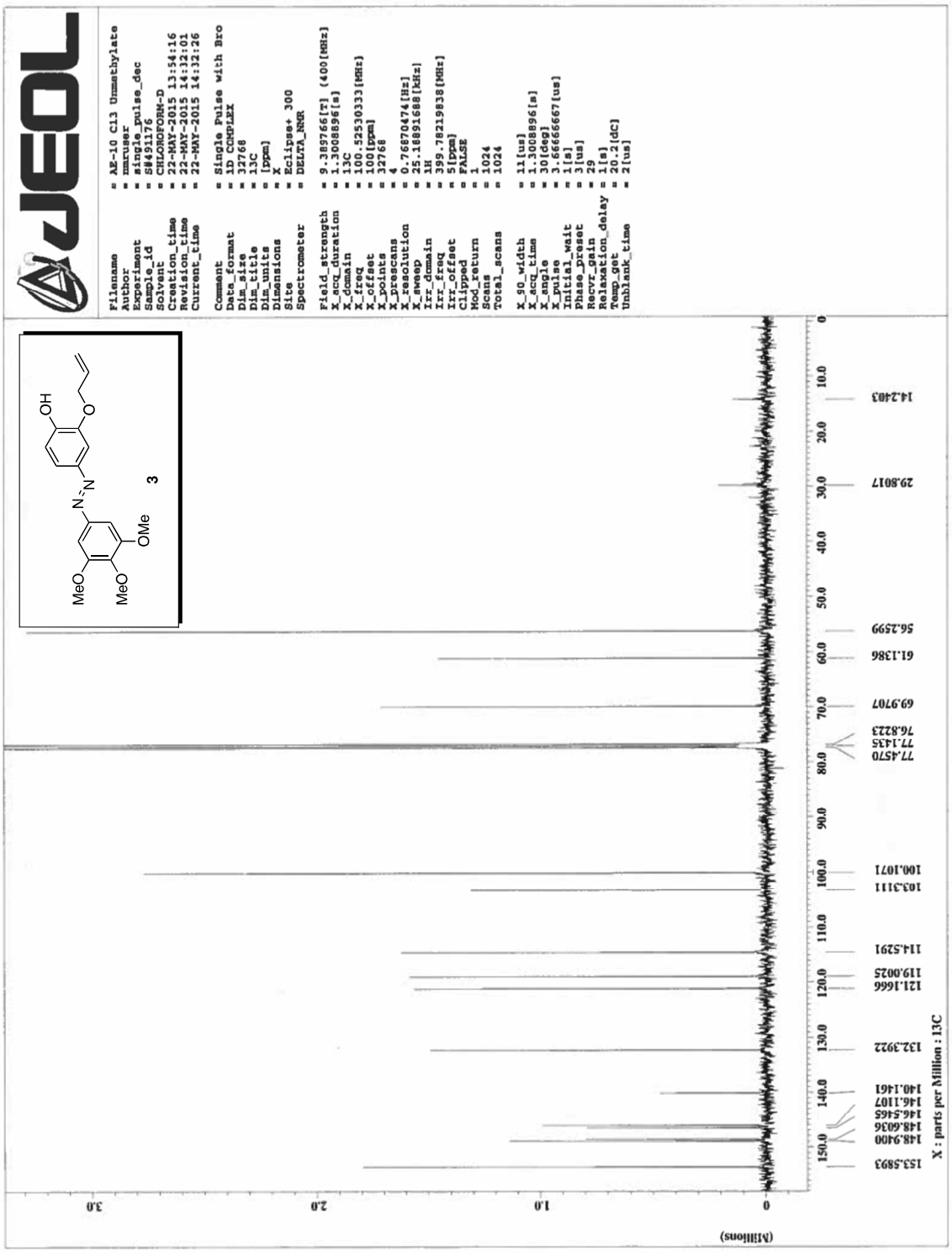


Figure S3. ^{13}C NMR of Compound 3 (125 MHz, $CDCl_3$).

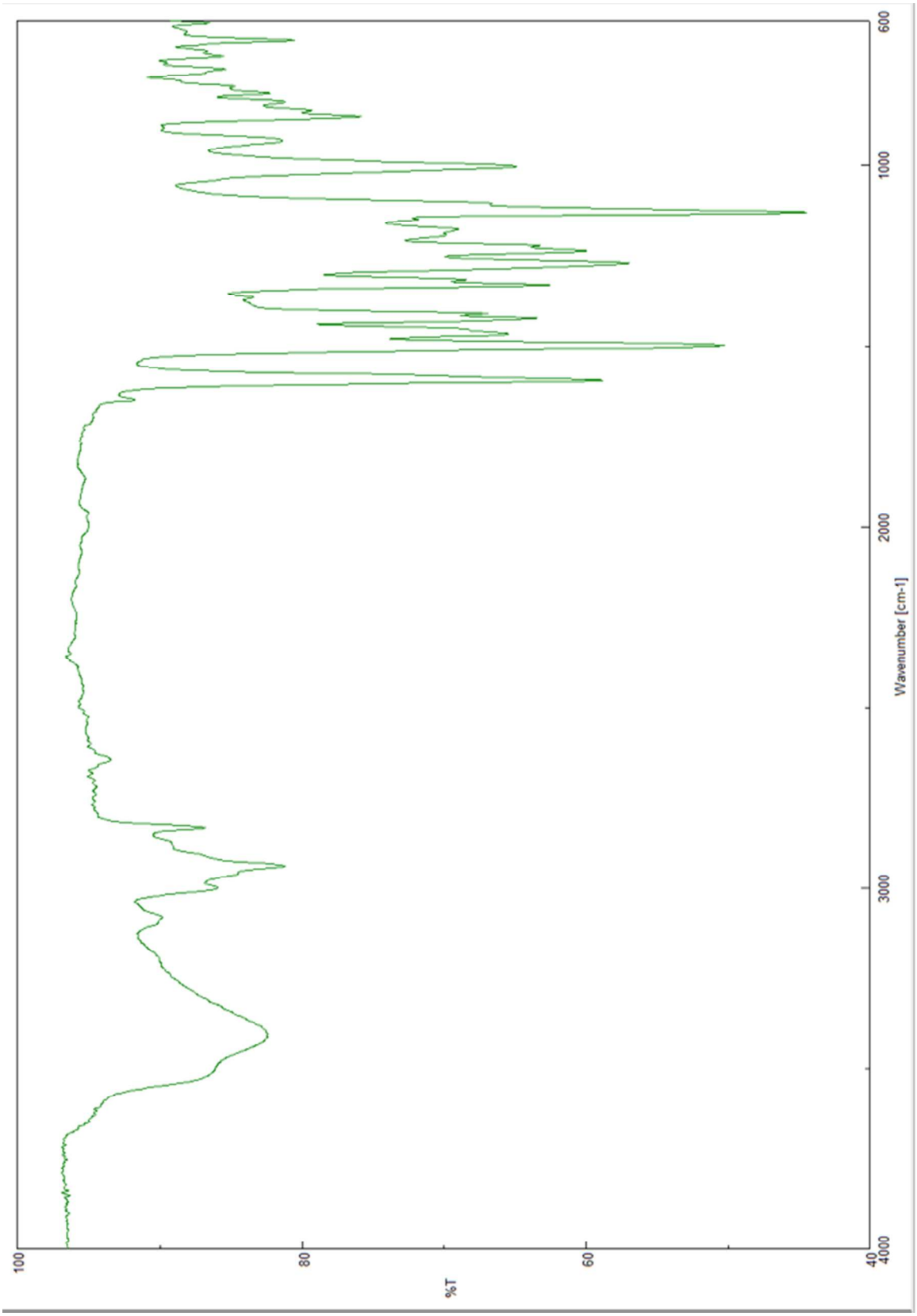
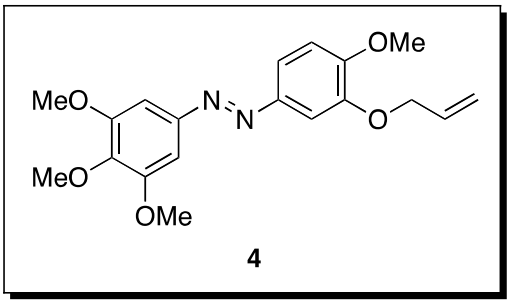


Figure S4. FTIR of Compound 3 (thin film).



4

Compound **4**. Compound 3 (0.30 g, 0.87 mmol) was dissolved in DMF (50 mL). Then K_2CO_3 (0.24 g, 1.7 mmol) was added and the solution was purged with nitrogen. Methyl iodide (0.14 mL, 2.2 mmol) was then added dropwise and the reaction was allowed to stir at room temperature for 3 days. The reaction was quenched with 2 M NaOH (30 mL) and the aqueous layer was extracted three times with CH_2Cl_2 (3x50 mL). The combined organic layer was washed three times with 3 M HCl (3x50 mL), dried over Na_2SO_4, and concentrated *in vacuo* onto diatomaceous earth. The desired product was isolated by automated flash chromatography with hexane:ethyl acetate mobile phase (0->20% EtOAc gradient over 20 minutes) to produce a light orange solid (260 mg) in 83% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm) 7.60 (dd, J = 8.8, 2.2 Hz, 1H), 7.49 (d, J = 2.2 Hz, 1H), 7.20 (s, 2H), 6.99 (d, J = 8.4 Hz, 1H), 6.13 (ddt, J = 17.2, 10.2, 5.3 Hz, 1H), 5.46 (ddd, J = 17.4, 3.2, 1.5 Hz, 1H), 5.32 (dd, J = 10.4, 1.3 Hz, 1H), 4.69 (dt, J = 5.4, 1.5 Hz, 2H) 3.95 (s, 9H), 3.91 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ(ppm) 153.6, 152.2, 148.64, 148.60, 146.8, 140.2, 132.9, 120.7, 118.6, 110.8, 103.8, 100.1, 69.8, 61.1, 56.3; IR (thin film) cm^{-1} 3085, 2999, 2937, 2837, 1591, 1501, 1461, 1415, 1350, 1333, 1314, 1260, 1232, 1120, 1006, 930, 855, 822, 808, 776, 687, 654.

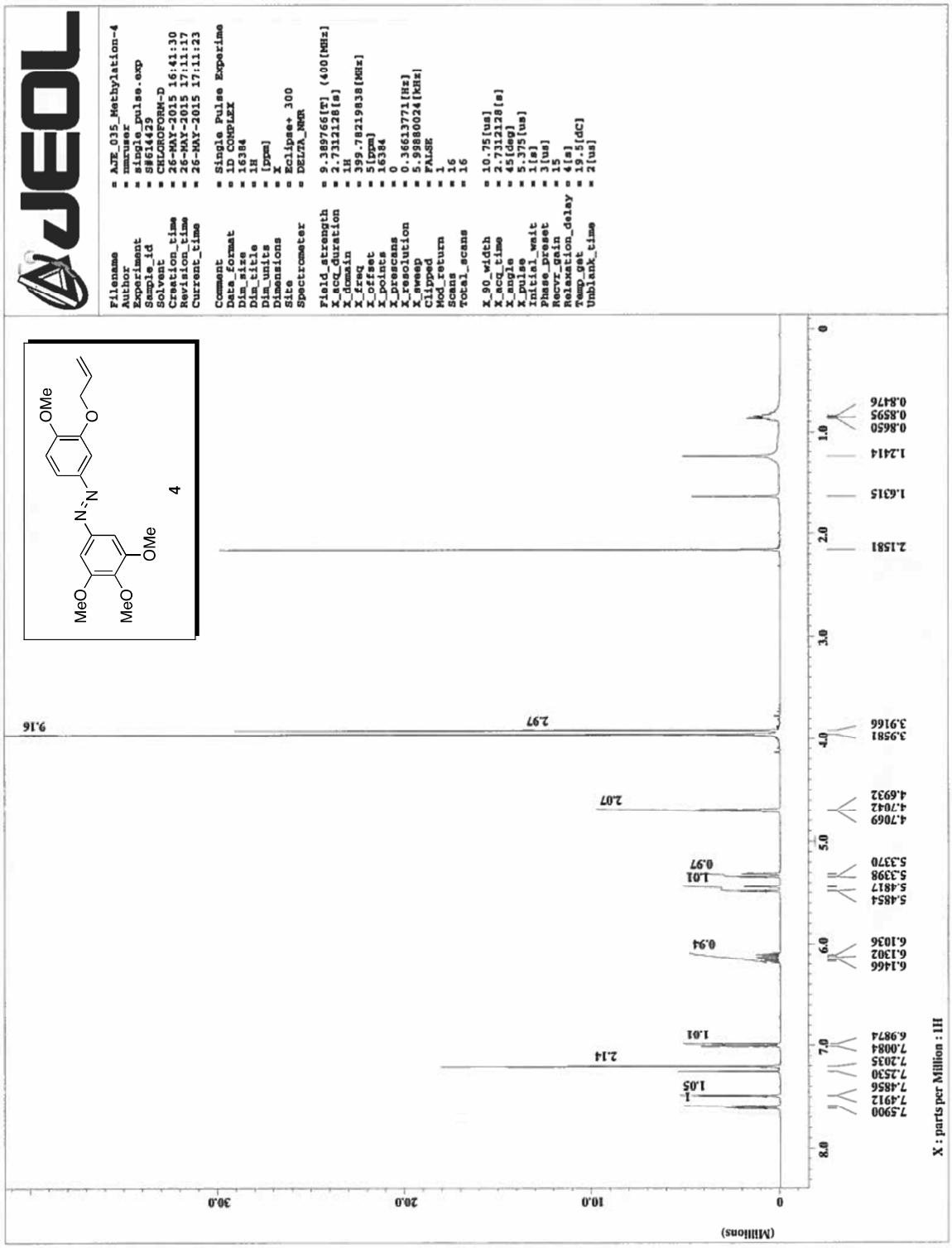


Figure S5. ^{1}H NMR of Compound 4 (400 MHz, $CDCl_3$).

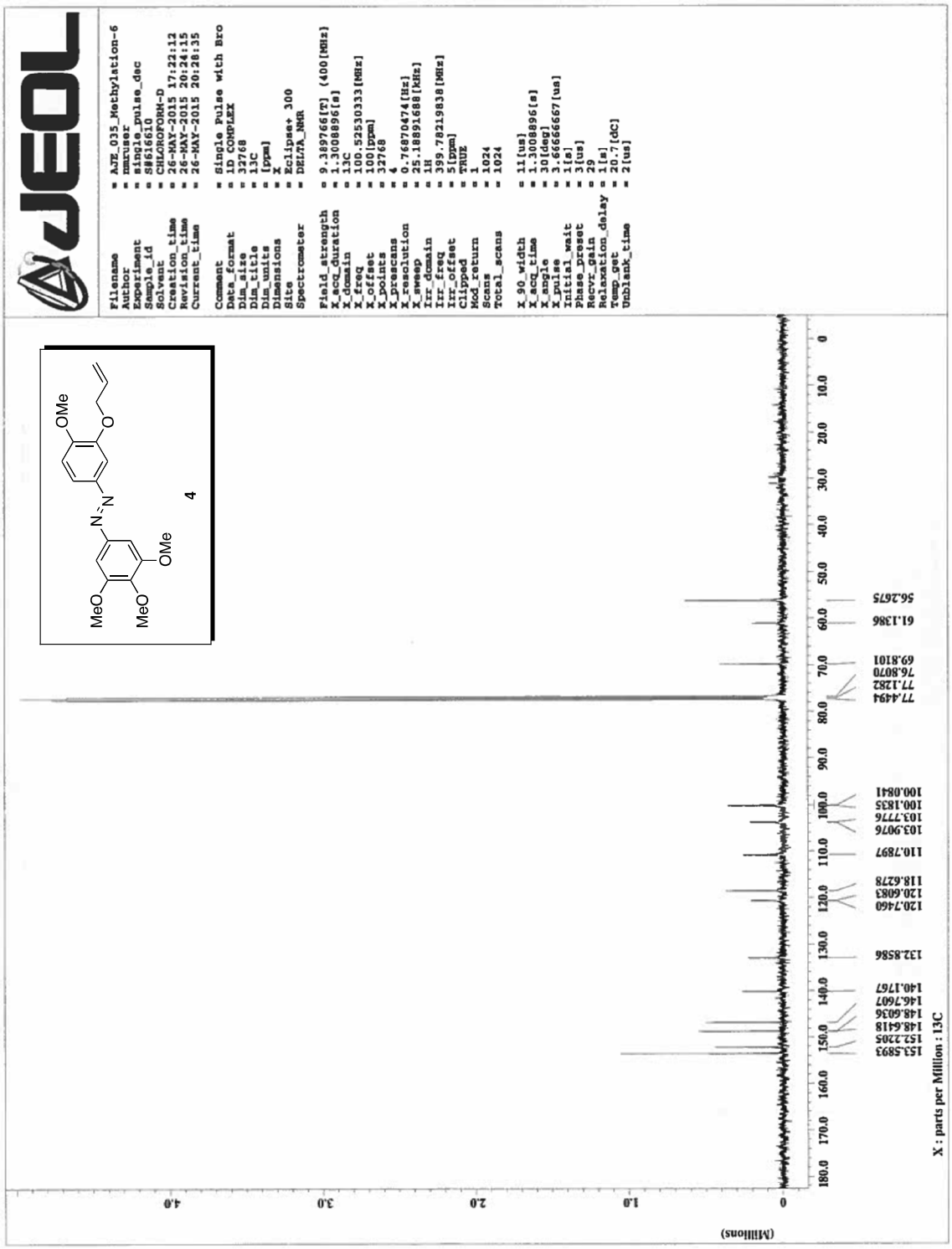


Figure S6. ^{13}C NMR of Compound 4 (125 MHz, $CDCl_3$).

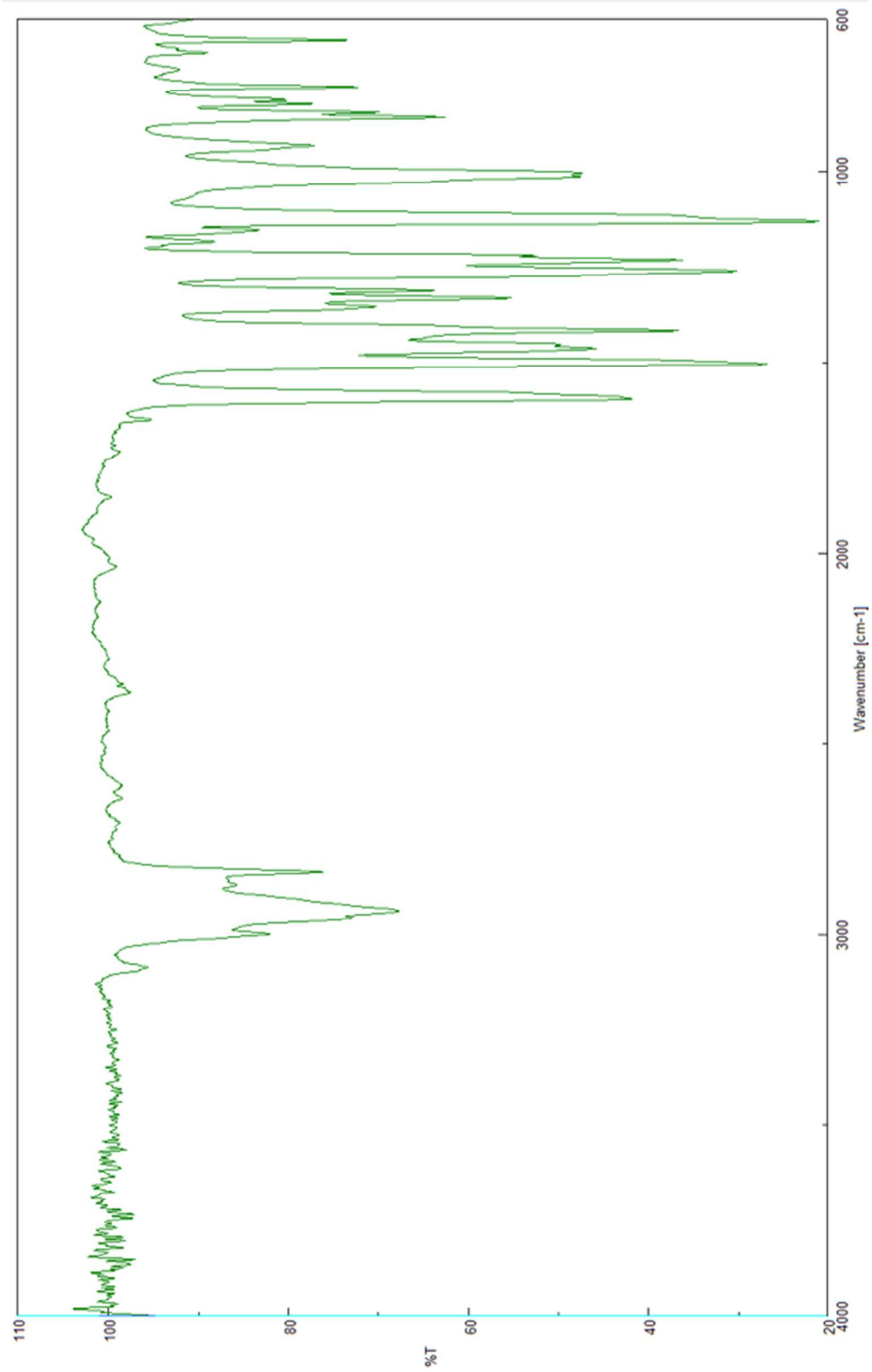
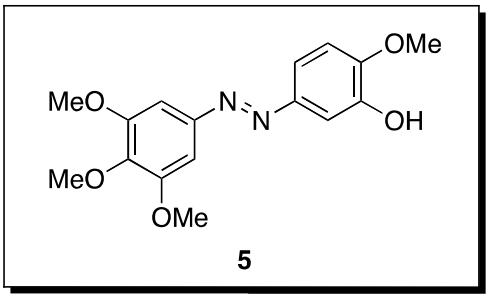


Figure S7. FTIR of Compound 4 (thin film).



5

Compound **5**. Compound **4** (0.23 g, 0.64 mmol) was dissolved in a 5:3 MeOH:CH_2Cl_2 solution (80 mL). The solution was then purged with nitrogen gas and $Pd(PPh_3)_4$ (0.015 g, 0.013 mmol) was added. After stirring for 5 minutes at room temperature, K_2CO_3 (0.53 g, 3.8 mmol) was added and allowed to stir for 90 minutes, during which time the reaction changed from yellow-orange to orange-red. After 90 minutes the reaction was concentrated. The residue was washed with 3 N HCl (50 mL). This aqueous layer was extracted three times with CH_2Cl_2 (3x50 mL). The combined organic layers were washed with brine, dried with Na_2SO_4 and concentrated *in vacuo* onto diatomaceous earth. The desired product was isolated by automated flash chromatography with hexane:ethyl acetate mobile phase (0->30% EtOAc gradient over 20 minutes) to produce an orange solid (159 mg) in 78% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm) 7.54-7.50 (m, 2H), 7.20 (s, 2H), 6.94 (d, J = 8.4 Hz, 1H), 5.88 (s, 1H), 3.94 (s, 6H), 3.92 (s, 3H), 3.91 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ(ppm) 153.6, 149.4, 148.6, 147.4, 146.3, 140.2, 119.2, 110.2, 106.1, 100.2, 61.1, 56.3, 56.2; IR (thin film) cm^{-1} 3001, 2937, 2836, 1590, 1498, 1462, 1411, 1329, 1310, 1269, 1237, 1219, 1173, 1127, 1026, 999, 971, 907, 873, 848, 805, 779, 762, 726, 650. HRMS calcd for $C_{16}H_{19}N_2O_5$ $(M+H)^+$ 319.1294, found $(M+H)^+$319.1292.

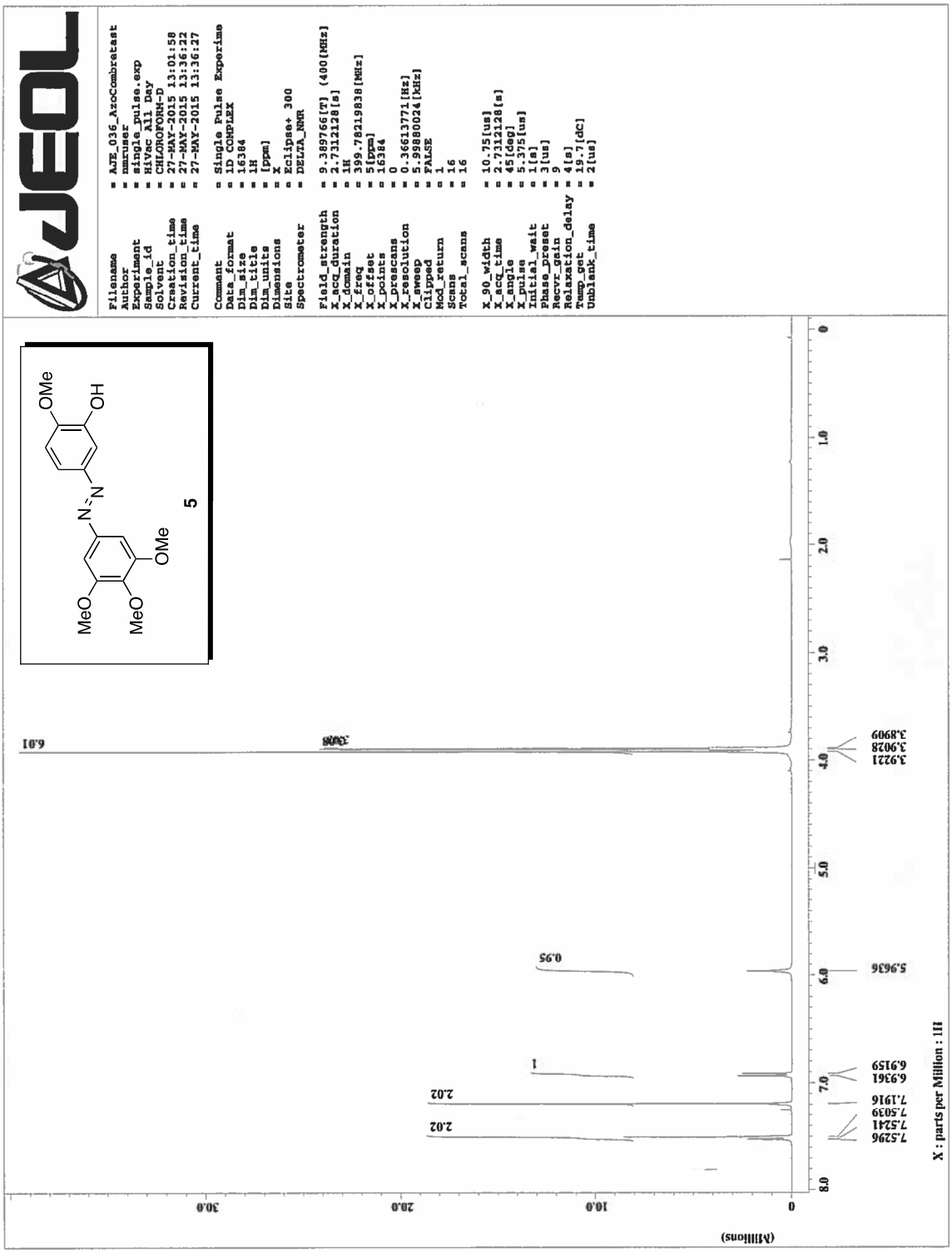


Figure S8. ^{1}H NMR of Compound 5 (400 MHz, $CDCl_3$).

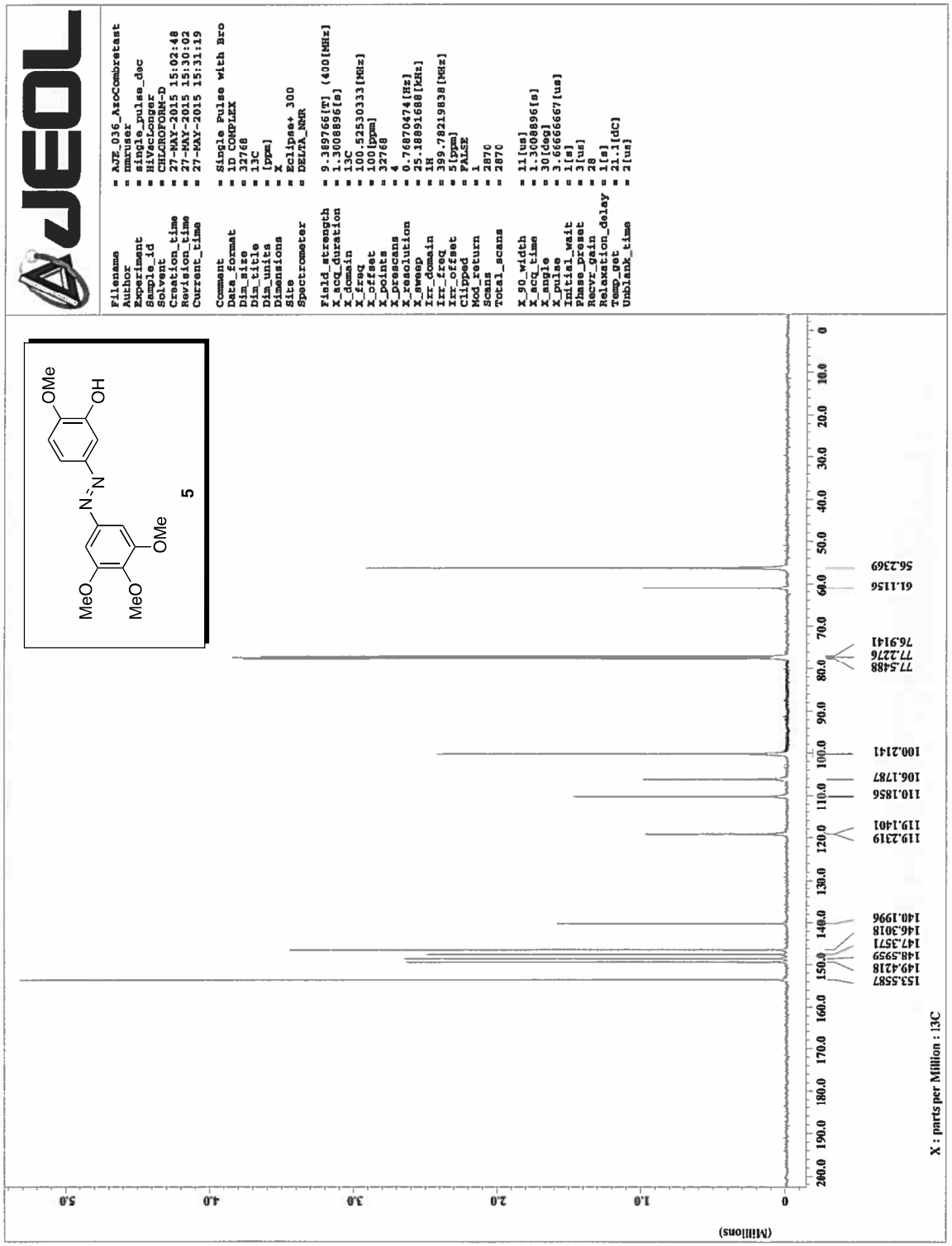


Figure S9. ^{13}C NMR of Compound 5 (125 MHz, $CDCl_3$).

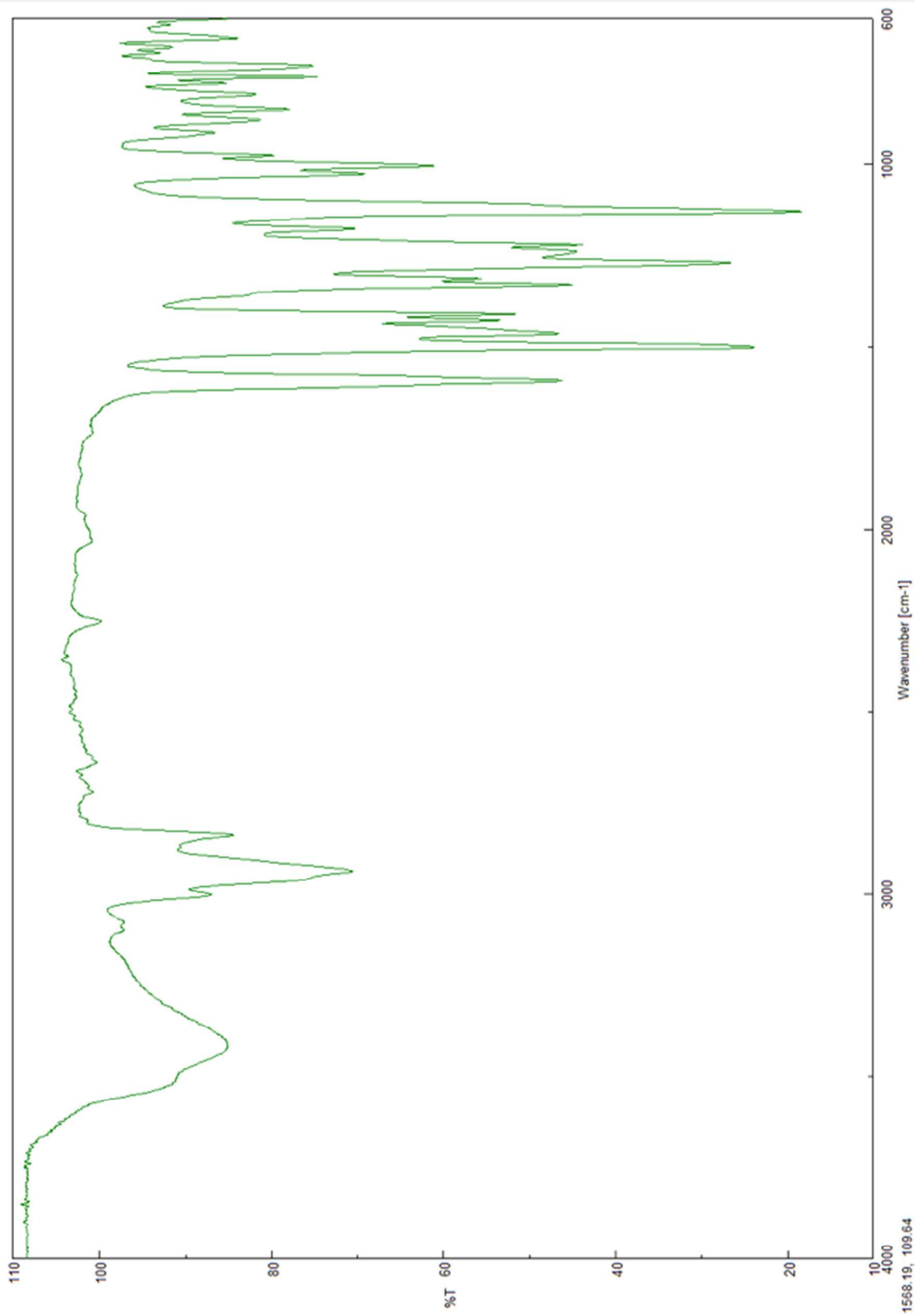


Figure S10. FTIR of Compound 5 (thin film).

Azo-CA4 Biological Testing General Information

Reagents were purchased from Fisher Scientific, Invitrogen, and Sigma Aldrich. Tubulin was obtained from three sources: Cytoskeleton Inc., Dr. Vale of UCSF, and Dr. Slep of UNC. All tubulin buffers were prepared from non-cytoskeleton sources following the cytoskeleton assay manual. HeLa cells were grown at 37 °C in EMEM supplemented with 10% FBS and penicillin/streptomycin. A UVP Model UVL Long wave TLC lamp was used for 365 nm irradiation, a 41 LED flashlight source hardwired to an Arduino board and 12V power supply was used for 400 nm irradiation, and an EverReady 6V EVML33AS flashlight was used for white light irradiation. Absorbance measurements were taken with either a Cary 50Bio UV-Vis spectrophotomerter using the Scan program, or a Thermo Multiskan plate reader.

Evaluating Isomerization

3 .3 μL of a 10 mM solution of our desired compound in DMSO was diluted in 1 mL DMSO to yield a 30 μM final concentration. The quartz cuvette containing the solution was then irradiated by close contact with the desired light. For tests to determine whether the light could penetrate the plastic well plate cover, a polystyrene petri-dish cover was placed between the light and the cuvette.

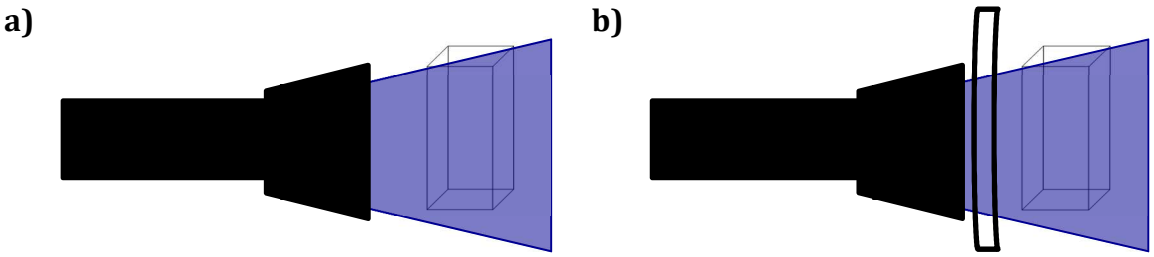


Figure S11. It is NOT Complicated! Diagram of irradiation setup demonstrating tests a) with, and; b) without the plastic petri dish cover.

Tubulin Polymerization Turbidity Measurements

Our assay was based on the Cytoskeleton Inc. porcine tubulin polymerization kit. A 96-well plate was pre-warmed to 37°C. 990 μL of general tubulin buffer (80 mM PIPES, 2.0 mM $MgCl_2$, 0.5 mM EGTA, pH 6.9) was combined with 10 μL of 100 mM GTP in water to make fresh G-PEM buffer for each round of tests. G-PEM buffer was used to dilute Vale lab tubulin from 25 mg/mL to 4 mg/mL and to prepare a 20x stock of each of the desired compounds from the 2 mM in DMSO stock. An equivalent concentration of DMSO only in G-PEM was used as a control. 10 μL of the compound of interest was incubated on the plate for two minutes, which allowed for activation by a light in light tests. After the incubation, 100 μL of tubulin was added, and tubulin polymerization was followed at 37 °C by absorbance. The spectrophotometer was placed inside a 37 °C incubator and instructed to pause for a minute between readings, and eject the 96-well plate. This allowed for the irradiation of the plate in the light tests, and no light was used for the dark tests.

Arduino Optimization

An arduino uno board was wired to a breadboard with a transistor to give a 5V output when the code was set to HIGH. This output was wired to a 41 LED flashlight. A battery was wired to a DC plug, and plugged into the board to power the setup. Three different battery types were used to run the system: 9V, 6AAA, and 6AA. Both codes included an adjustable off time for the light (B.1 and B.2), and the second (B.2) included a "sleep" time for the arduino to conserve battery.

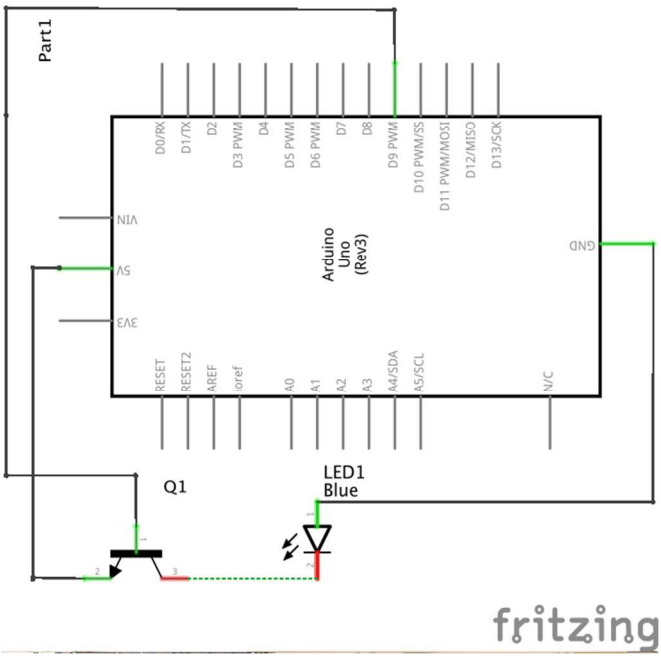
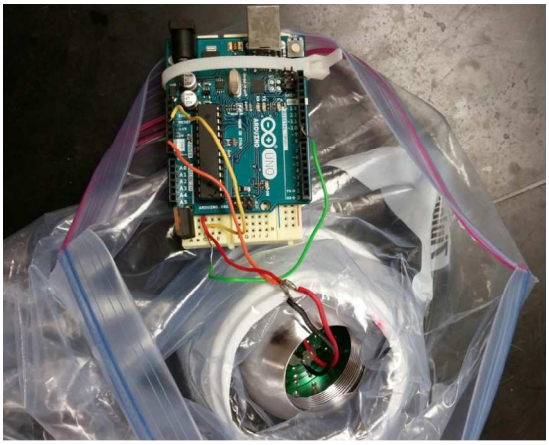


Figure S12. Arduino circuit board diagram.

The setup was sealed in a plastic bag, and the edges were parafilmed, taped and sealed. The bag was sterilized with ethanol and the light was placed inside a sterilized yogurt cup. The setup was placed in the incubator and set over the polystyrene 96-well plate as shown below.

a) 

b) 

Figure S13. a) Internal Arduino setup, and; b) and incubator Arduino setup. For irradiations exceeding 12 hours, light sources were hard wired to a 12V AC power source.

First Generation Arduino Program

```
/*Ashton's SMP*/

// Pin 9 is where we will have your flashlight hooked up to
int led = 9; // the name is led, and it is in pin 9

// the setup routine runs once when you press reset:
void setup() have the functional enzyme necessary  to

// the loop routine runs over and over again forever:
void loop() {
  digitalWrite(led, HIGH);   // turn the LED on (HIGH is the voltage level)
  delay(10000);               // wait for 10 seconds in milliseconds
  digitalWrite(led, LOW);    // turn the LED off by making the voltage LOW
  delay(3590000);             // wait for 59 mins 50secs  in milliseconds
}
```

Arduino Code for Low Voltage Delay.

Because the original Arduino code was not suited for low voltage conditions since it consumed batteries far too quickly to be useful for the long incubation times needed, a second Arduino code was written with a low voltage delay.

```
/* Ashton's SMP */

// Pin 9 is where we will have your flashlight hooked up to
int led = 9; // the name is led, and it is in pin 9

//Variable to keep track if watchdog timer
volatile int f_wdt=0;

//Headers to allow use of watchdog timer
#include <avr/sleep.h>
#include <avr/power.h>
#include <avr/wdt.h>

// the setup routine runs once when you press reset:
void setup() {
//Nick's Contribution
  /*** Setup the WDT ***/

  /* Clear the reset flag. */
  MCUSR &= ~(1<<WDRF);

  /* In order to change WDE or the prescaler, we need to
   * set WDCE (This will allow updates for 4 clock cycles).
   */
  WDTCSR |= (1<<WDCE) | (1<<WDE);

  /* set new watchdog timeout prescaler value */
  WDTCSR = 1<<WDP0 | 1<<WDP3; /* 8.0 seconds */

  /* Enable the WD interrupt (note no reset). */
  WDTCSR |= _BV(WDIE);

  // initialize the digital pin as an output.
  pinMode(led, OUTPUT);

}
//THIS IS THE PART YOU EDIT
// the loop routine runs over and over again forever:
void loop() {
   if(f_wdt==0){            //Number of 8 second sleeps before turning on the light
```

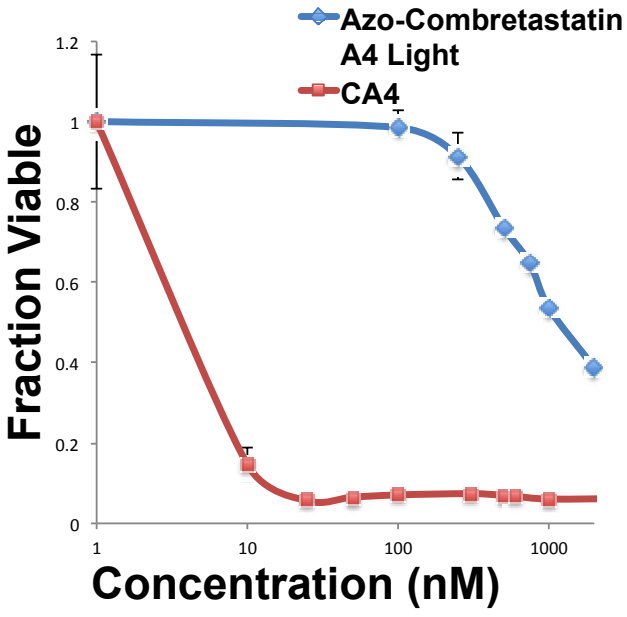
```
   /* Re-enable the peripherals. */
   power_all_enable();       //Powers outputs
   digitalWrite(led, HIGH);   // turn the LED on (HIGH is the voltage level)
   delay(10000);              // wait for 10 seconds in milliseconds
   digitalWrite(led, LOW);    // turn the LED off by making the voltage LOW
   f_wdt=240;                 //Reset counter
   enterSleep();             //Set arduino to sleep
  }
  else{                    //If not enough time has passed go back to sleep
   enterSleep();
  }
}

void enterSleep(void)      //Put arduino to sleep for 8 seconds
{
 set_sleep_mode(SLEEP_MODE_PWR_DOWN);   /* EDIT: could also use
SLEEP_MODE_PWR_SAVE for better opperation. */
 sleep_enable();

 /* Now enter sleep mode. */
 sleep_mode();

 /* The program will continue from here after the WDT timeout*/
 sleep_disable(); /* First thing to do is disable sleep. */
}

ISR(WDT_vect){
 f_wdt=f_wdt-1;  //Increment number of times arduoino has gone to sleep for 8
seconds
}
```

MTT Cytotoxicity Assay

HeLa cells were plated at 2,000 cells/well in a 96-well plate and left to adhere at 37°C overnight (5% CO_2) in DMEM supplemented with 10% FBS as well as penicillin/streptomycin. 2 mM stock solutions of azo-CA4 (5) were prepared in DMSO, and diluted to the desired 10x concentration in PBS. Compounds were further diluted to 1x in the wells containing HeLa cells, and incubated for the time indicated. Negative controls used the highest concentration of DMSO, always <1% overall. After the drug incubation, period, a 10x (5 mg/mL) MTT solution was diluted to 1x in the wells and left to incubate for 2-4 hours. After the incubation the media was removed, the formazan crystals were dissolved in DMSO and absorbance measured at 570 nm.

MTT Analysis

The EC_{50} value for azo-CA4 in the dark well exceeded 100 µM. We were unable to achieve <50% cell survival at all concentrations tested (up to 100 µM).



General procedure for light microscopy cell viability screen:

HeLa cells were plated at a density of $3x10^4$ cells/well in 2 mL of DMEM (10% FBS, containing penicillin/streptomycin) in a 24-well plate. Cells were allowed to adhere overnight then incubated with compound (azo-CA4 500 nM, CA4 50 nm, DMSO equivalent concentration) for 12 hours with or without isomerizing (400 nm, 12V) light for 12 hours using the Arduino light pulse program (10 s irradiation every 30 minutes). Cells were imaged with the BioRad ZOE™ Fluorescent Cell Imager using white light.

References:

During the preparation of this manuscript, a competing publication was released:

‡Borowiak, M.; Nahaboo, W.; Reynders, M.; Nekolla, K.; Jalinot, P.; Hasserodt, J.; Rehberg, M.; Delattre, M.; Zahler, S.; Vollmar, A.; Trauner, D.; Thorn-Seshold, O., Photoswitchable Inhibitors of Microtubule Dynamics Optically Control Mitosis and Cell Death. Cell, 2015, 162, (2), 403-411.