SUPPORTING INFORMATION FOR:

Phage-Assisted Continuous Evolution and Selection of Enzymes for Chemical Synthesis

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Pages: 65
Figures: 48
Tables: 4

Table of Contents

I. Materials and Instruments

Greiner Bio-One polypropylene 96-well plates (product number 651201) or Fisherbrand 96-well deepwell polypropylene plates (product number 12-566-121) were purchased from Fisher. Corning-costar 96-well black wall, clear bottom plates (product number 3631) were purchased from Fisher.

Antibiotics were purchased from Gold Biotechnology and were prepared as 1000x stock solutions. Sodium acrylate was purchased from Combi-Blocks (product number QC-1489-005). IPTG was purchased from Gold Biotechnology (product number I2481). Estradiol was purchased from Combi-Blocks (product number QA-4119). Abscisic acid was purchased from Biosynth International (product number A-0120). Stock solutions (50 mM) of ligand were prepared at 50 mM in either DMSO (estradiol and abscisic acid) or water (sodium acrylate and IPTG) and stored at -20 °C until use. Acrylate ester substrates and ethyl 3,3-dimethylacrylate were purchased from Sigma-Aldrich and used without further purification. Estradiol ethers were synthesized as previously reported (PMID: 11141094).

Phusion polymerase was expressed, purified, and titrated prior to use[1]. Taq DNA polymerase and Q5 High-Fidelity DNA polymerase was purchased from New England Biolabs. LBD and SRC RID3 genes were synthesized by Twist Bioscience. The genes for ABI and PYL were gifts from Professor Fu-Sen Liang. The genes for AcuR was a gift from George Church (pJKR-L-acuR, Addgene plasmid #62568; http://n2t.net/addgene:62568; RRID:Addgene_62568). The above genes were integrated into a plasmid containing *gIII* translationally coupled to the bacterial lux operon. Links to all sequences are provided in **Supplementary Table S1** or as Genbank sequence files, which include annotated sequence maps, full vector sequences, and primer annotations.

Luria broth (LB) media and Super Optimal broth with Catabolite repression (SOC) media were purchased from Research Products International and Davis rich media for PACE or PACS was prepared as previously described[2]. *E. coli* 10β were purchased from New England Biolabs and *E. coli* S1030 cells[2] were courtesy of the Liu lab, Harvard University. Restriction enzymes, Antarctic Phosphatase, and T4 DNA ligase were purchased from New England Biolabs. DNA clean & concentrator kits were purchased from Zymo Research (product number D4013).

Luciferase and optical density measurements were performed using a Synergy Neo2 Hybrid Multi-Mode Reader (BioTek). For UHPLC-MS analysis, an Agilent system equipped with a 1290 Infinity II Multisampler (dual-needle configuration), a 1290 Infinity II high-speed pump, a 1260 Infinity II

diode array detector, and a 6135X single quadrupole mass spectrometer with an Agilent Jet Stream ESI source was used for both low-throughput analysis and the qualitative MISER screen. For UHPLC analysis, a similar Agilent system was used, except the autosampler used was single-needle configuration. For both UHPLC-MS and UHPLC, low-throughput analysis was performed on an Eclipse Plus C18 2.1x5mm guard column with a 1.8 µm pore size (part number 821725-901) connected to a ZORBAX rapid resolution C18 column (part number 959757-902). MISER analysis was done using only the guard column.

II. General methods

A. Cloning and sequencing.

All plasmids were constructed by Gibson assembly from PCR products generated using Phusion Polymerase or Q5 High-Fidelity DNA Polymerase (NEB). PCR reactions and thermocycling conditions were performed according to NEB's recommended protocols. Annealing temperatures were determined with the NEBTm Calculator. Phage were cloned by Gibson assembly of BS2 into a previously optimized[3] SP phage backbone and transformed into previously developed 1059 cells[2], which supply *gIII* in an activity-independent manner. After overnight growth of phage in media, the supernatant was isolated and plaque assays were performed on 1059 cells. Single plaques were grown overnight and sequenced to identify phage samples with the correct insert. All plasmids and phage were sequenced by the University of Chicago Comprehensive Cancer Center DNA Sequencing and Genotyping Facility. Maps for each plasmid are shown in **Supplementary Fig. S1** and are described in **Supplementary Table S1**, which includes links to fully annotated sequence maps, full vector sequences, and primer annotations.

B. Biosensor screening in *E. coli* for small molecule detection.

10β cells were transformed by heat shock with either AcuR, IPTG, estradiol, or ABA biosensors (**Table S1 and Figure S1**). Single colonies were grown to saturation overnight at 37 °C and then each well of a 96-well deep well plate containing 0.185 mL of LB with antibiotics and 500 µM control small molecule substrate or ester substrate was inoculated with 20 µL of the overnight culture. After growth with shaking for 6 h at 37 °C, 150 µL of each culture was transferred to a 96-well black wall, clear bottom plate (Costar), and luminescence and OD_{600} were measured on a Synergy Neo2 Hybrid Multi-Mode Reader (BioTek). The data were analyzed by dividing the luminescence values by the background-corrected OD_{600} value. All values were then normalized to the emission of cells expressing each biosensor without small molecule, which was assigned

an arbitrary value of 1 to allow for values from each luminescence plot to be compared to each other. All experiments were performed in four technical replicates.

C. Phage growth assays.

S1030 cells were transformed by heat shock (42 °C, 45 sec) with either AcuR, IPTG, estradiol, or ABA biosensors. Single colonies were grown to saturation overnight at 37 °C and then each well of a 24-well deep well plate containing 0.375 mL of LB with antibiotics and 1.5 mM small molecule ester substrates or 500 μM control substrates was inoculated with 125 μL of the overnight culture. After growth with shaking for 2 h at 37 °C, 10 μL of BS2 phage (1 x 10^5 PFU/μL) was added to each well. Cultures were then grown for an additional 5-6 h at 37 °C with shaking and supernatant was then isolated for plaque assays on 1059 cells to calculate phage titers.

III. Phage assisted continuous evolution (PACE) and selection (PACS).

A. General PACE setup and methodology

PACE was performed utilizing a modified version (see Figure 5B) of the previously described PACE method[4]. S1030 cells were transformed by electroporation with the ABA biosensor plasmids (8-15 and KJ223) and mutagenesis plasmid MP6. A 6 mL starter culture was grown overnight in LB supplemented with antibiotics and 10 mM glucose. Chemostats (125 mL sterile flasks) containing Davis rich media were inoculated with 5 mL of starter culture and grown at 37 °C with magnetic stir-bar agitation. At approximately OD_{600} 0.5, 30 mL of chemostat culture was pumped into the holding tank (50 mL sterile flask) with 500 μM ABA (evolutionary drift) or 1.5 mM ABA ester (catalysis-dependent evolution) and was pre-incubated for 3 h. Fresh Davis rich media was then pumped in to the chemostat at 60-80 mL/h, with a waste needle set at ~75 mL. To initiate the evolution, 30 μL of BS2 phage were seeded in fresh lagoons (50 mL sterile flasks) and waste needles were set to maintain the lagoon volume at ~20 mL. Host cell cultures were flowed in from the holding tank at ~10 mL/h. Arabinose (10% w/v in water) was added directly to lagoons via syringe pump at 1.0 mL/h to induce mutagenesis. ABA (evolutionary drift) or ABA ester (catalysis-dependent evolution) was added directly to the holding tank via syringe pump at 1.0 mL/h to maintain a final concentration of 250 μM ABA (day 1) – 100 μM ABA (day 2) or 750 μM ABA ester in the holding tank. Waste needles were set to maintain the lagoon volume at 20-25 mL, and host cell cultures were flowed in at 10-12 mL per hour. A lagoon sample was taken from the lagoon every 24 h, centrifuged, and the supernatant was stored at 4 °C. The phage sample was subcloned into KJ89 for analysis with the luciferase reporter system.

For the competition PACE experiment, 30 μL of a 500:1 mixture of human rhinovirus (HRV) protease to BS2 phage was used to inoculate the lagoons. Lagoon samples were taken every 6-12 h and miniprepped for analysis by PCR.

B. BS2 Library Generation.

Phage vector backbone was amplified overnight in 1059 cells. The BS2 insert was prepared by using error prone PCR (epPCR) to introduce mutations into the gene. Briefly, 50 μL epPCR reactions were set up with the following final concentrations: 5 ng BS2 template plasmid, 0.2 μM forward primer, 0.2 μM reverse primer, 1x standard NEB Taq buffer, 0.2 mM dNTPs, 400 μM $MnCl_2$, and 0.025 U/μL NEB Taq polymerase.

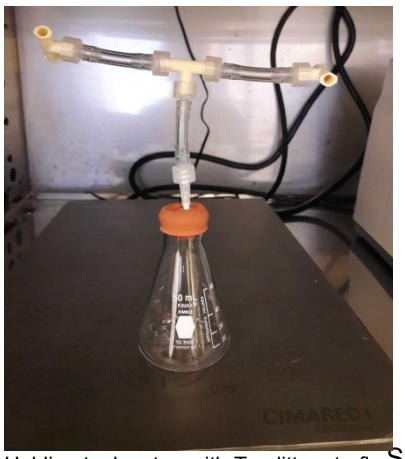
Fwd: 5' ctttttttcgcgccagaaggagaccGCTAGCATGACTCATCAAATAGTAACGACTC

Rev: 5' cgattgagggagcatgttgaaaatctccaGGATCCTCAGGACCCTTCTCCTTTTGAAG

Thermocycling conditions were performed according to NEB's recommended protocols. The PCR product was digested with 1 μL NEB DpnI enzyme in the PCR reaction buffer for 2 h at 37 °C and then purified with a Zymo clean and concentrator kit. Insert and vector were restriction enzyme digested with NEB NheI enzyme and NEB BamHI enzyme using the recommended NEB double digest protocol. The vector was then treated with NEB Antarctic Phosphatase. Ligations were performed at 16 °C for 16 h using NEB T4 DNA ligase following the manufacturer's protocol. The ligation reactions were purified using a Zymo DNA clean and concentrator kit. The library (1 μg total DNA) was then electroporated (1800 V, 5 ms, 0.2 cm cuvettes, 50 μL cells) into 350 μL of electrocompetent 1059 cells. Cells were recovered with 25 mL of SOC media for 1 h and then used to inoculate 250 mL of super broth. The phage library was then cultured at 37 °C with shaking for 8 h to allow for phage library growth. The supernatant was collected by centrifugation and sterile filtration. Plaque assays were performed on 1059 cells. Single plaques were sequenced to confirm epPCR error rate.

C. Phage assisted continuous selection (PACS).

PACS was performed in a similar manner as the PACE experiments, with some differences. S1030 cells were transformed by electroporation (1800 V, 5 ms, 0.2 cm cuvettes, 50 μL cells) with only the ABA biosensor (8-15 and KJ223). For BS2 library screening, four separate lagoons were inoculated with 500 μL of the BS2 phage library. The line from the holding tank was split with two T-splitters to maintain the lagoon volumes at 20-25 mL with a host cell culture flow



Holding tank setup with T-splitters to flow into multiple lagoons.

rate at 10-12 mL per hour. Samples were collected from the lagoons at 24 h.

D. Post PACS library preparation.

After subcloning the PACS library from phage samples back into pET28, the library plasmids were transformed into BL-21(λDE3) via electroporation. 900 of the resulting colonies along with 3 positive controls per plate consisting of WT-BS2 and 3 negative controls per plate of empty pET28 were then arrayed into ten 96 well plates with 300 µL LB with 50 ng/µL of kannamycin for a total of 960 individual samples. These colonies were incubated overnight, and the following day 30 µL of overnight volume was used to innoculate 1 mL TB with 50 ng/µL kannamycin. The variants were grown at 37 °C until the OD600 was between 0.6 and 0.8, at which point esterase expression was induced with 10 µL of a 10 mM stock solution of IPTG in phosphate buffer (50 mM, pH 7.4) for a final concentration of 100 µM. Expression was allowed to continue for 20 hours at 30 °C, at which point the cells were pelleted via centrifugation at 3600 RPM for 15 minutes. The supernatent was removed, and the cells were stored at –80 °C for at least two hours or until the library was screened.

IV. Biocatalysis methodology

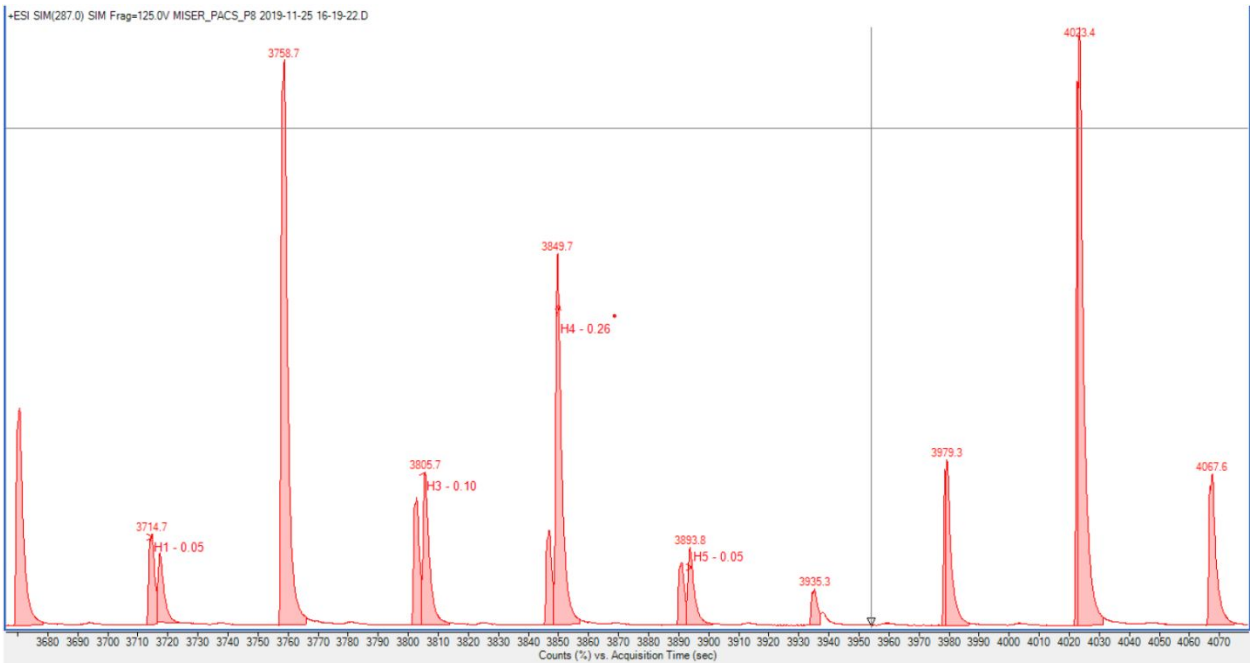
A. General procedure for library lysis.

The frozen 96-well plates were removed from the –80 °C freezer and allowed to warm for 10 minutes at room temperature. To each well in the 96 well plate, 100 µL of lysis buffer (0.75 mg/mL lysozyme, 50 mM phosphate, pH 7.5) was added, and the cells were resuspended by vortexing until homogenous. A non-breathable plate seal was affixed to each 96-well plate, and the cells were lysed at 37 °C, 250 rpm for 45 minutes. Once the lysis was complete, the samples were then flash frozen with liquid nitrogen and then gradually allowed to warm to room temperature. Once thawed, 10 µL of DNAse buffer (0.1 mg/mL DNAse I in 50 mM phosphate, pH 7.4) was added to each well and the samples were incubated at 37 °C for an additional 15 minutes. After DNAse treatment was finished, the cell lysate was centrifuged at 3600 rpm, 4 °C for 15 minutes to pellet cell debris. Cell lysate was then used immediately for bioconversions.

B. MISER (Multiple Injections in a Single Experimental Run)[5] screening.

For all LC-MS usage, mobile phase A was water with 0.1% formic Acid, and mobile phase B was acetonitrile with 0.1% formic acid. MISER screening was performed on by LC-MS using an Agilent system equipped with a 1290 Infinity II Multisampler (dual-needle configuration), a 1290 Infinity II high-speed pump, a 1260 Infinity II diode array detector, and a 6135X single quadrupole mass

spectrometer with an Agilent Jet Stream ESI source. A guard column was used to separate buffer from the analyte of interest, and the flow was directly analyzed by the mass-spec, bypassing the UV-Vis detector in the LC-MS system. A flow rate of 1 mL/min with an isocratic method of 32%B with a 42 second delay between injections was used for all MISER screening. The method was setup as an FIA method using the Chemstation software, and the peak height of the resulting abscisic acid product was used as a qualitative measure of conversion. MassHunter software was used to visually inspect and label the chromatograms. To monitor the product ion, SIM was used for the sodium adduct of the product peak (M+Na)+ corresponding to an m/z of 287. An example of the output from the MISERgram is included below.



SI Figure: MISERgram cut-out. Presence of the second peak indicates product.

C. High-throughput MISER screen and comparison to low-throughput UHPLC.

To compare the reliability of the MISER screen to a low-throughput UHPLC screen, the first two plates of post-PACS samples were grown and lysed as described above. 5 µL of a 11.25 mM stock of **4b** in DMSO was delivered to a 96-well microtiter plate, followed by 20 µL of phosphate buffer (50 mM, pH 7.4). Reactions were initiated by the addition of 50 µL of lysate, and then the microtiter plate was covered by a non-breathable plate seal and the reactions incubated for 90 minutes without stirring. After 90 minutes time, the reactions were quenched via the addition of one volume of methanol. The resulting precipitated protein was separated via centrifugation at 3600 rpm, 4 °C for 15 minutes. The samples were then further diluted via addition of 150 µL water to 30 µL of quenched bioconversion and filtered through a 0.22 µm filter plate (Agilent product

number 201276-100) into a polystyrene microtiter plate. These plates were then heat-sealed and submitted for analysis by the MISER screen described above. After the high-throughput analysis by LC-MS, the same plates were then removed from the autosampler and re-filtered through the 0.22 μm filter plates and analyzed by low-throughput UHPLC-MS using method A. To provide a qualitative measurement of activity in the high-throughput MISER approach, the peak height of the abscisic acid product peak was used. For the low-throughput UHPLC data, the qualitative measure was the area under the curve of the product peak over the total area under the curve of the product and starting material peaks. To normalize the data between the two different analyses, the values for each well were normalized to the intra-plate average of the WT values. Microsoft Excel was used for data processing, and Graphpad Prism used for data visualization.

D. Creation and screening of the PACS compilation plate.

After screening the 10 post-PACS plates via MISER, active variants were pooled into a single 96-well plate. This plate was stamped out twice, and secondary plates were inoculated and induced as described per the general library preparation. For the bioconversions with **4b**, 5 μL of a 11.25 mM stock solution of **4b** in DMSO was added to a microtiter plate and diluted with 25 μL of phosphate buffer (50 mM, pH 7.5). The reactions were initiated with the addition of 45 μL of clarified lysate, and these reactions were allowed to incubate for 90 minutes at room temperature without shaking. After 90 minutes, the reactions were quenched via addition of one volume of methanol, and the precipitated protein was sedimented via centrifugation at 3600 rpm for 15 minutes at 4 °C. The reactions were then diluted with 30 μL of lysate and 150 μL of water, then analyzed by LC-MS. UV-absorbance at 254 nm was used to provide a qualitative measure of conversion. Data processing was done using the ChemStation software and Microsoft Excel, and the data was visualized using GraphPad Prism.

To screen the compilation plate for substrate **5**, the same procedure was followed, except 5 μL of a 100 mM stock solution of substrate **5** was diluted in 45μL phosphate buffer, followed by the addition of 50 μL clarified lysate. These reactions were then incubated for 60 minutes while shaking on a Heidolph Titramax 1000 plate shaker at 750 rpm. They were processed the same as the reactions above, and analyzed by UHPLC using method C. The plate was screened in triplicate, and the top five mutants expressed for purified reactions with substrate **5**.

E. Purification and characterization of PACS hits with abscisic acid esters 4a-4d and 3,3-dimethylacrylate esters 5, 6, 7, 8.

The top 8 variants for which good sequencing could be obtained were grown as overnights in 10 mL LB with 50 ng/μL kannamycin. 5 mL of this overnight culture was used to innoculate 250 mL of TB + 50 ng/μL kannamycin and the cells were grown at 37 °C shaking at 250 rpm until the OD600 reached 0.8, at which point the temperature was reduced to 30 °C and protein expression was induced with 25 μL of a 1 M stock solution of IPTG for a final concentration of 100 μM. These cultures were allowed to continue incubating for 20 hours at 250 rpm, at which point the cells were transferred to cell buckets and centrifuged at 3600 rpm, 4 °C for 20 minutes. The supernatent was removed and the cell pellets were stored at –20 °C until ready for purification. To begin purifying the protein, each cell bucket was resuspended in 40 mL of phopshate buffer (50 mM, pH 7.5), and the resuspended cell pellets were transferred to a 50 mL centrifuge tube. These were immediately placed on ice, and the cells were lysed by sonication using a QSonica S-4000 with a 0.5" horn at 40W using 1 min on/1 min off cycles for 5 min total cycle time. The cells were then centrifuged in a high-speed rotor at 15,000 rpm for 30 minutes at 4 °C. The clarified lysate was then purified via IMAC using Nickel-NTA resin. Buffer exchanging was done by dialyzing the eluted protein sample in phosphate buffer (50 mM, pH 7.5) overnight. The next day, the samples were concentrated in a Amicon ultracentrifugation column (10 kDa MW cutoff) and the buffer was diluted with 10 % glycerol to act as a cryoprotectant. Protein concentration was determined by the absorbance at 280 nm with the extinction coefficient calculated from the protein sequence using the Expasy Protein Parameters website. Purified enzymes were stored at –80 °C until used.

To begin the bioconversions with **4a-4d**, 100 μM stock solutions of each enzyme were prepared. 1.88 μL of 30 mM 4a-4d were added to a microtiter plate, followed by 69.4 μL of phosphate buffer (50 mM, pH 7.5). Reactions were initiated by the addition of 3.75 μL purified enzyme to the reactions, at which point the plates were sealed with a non-breathable plate seal and the reactions were left at room temperature with no shaking for one hour. After this point, one volume of quench solution (200 μM 2-Acetamidophenol in methanol) was added and the reactions were processed as described for the lysate reactions above. Reactions were analyzed by UHPLC monitored at 280 nm using method B. Product concentration was determined using a calibration curve.

Bioconversions with substrates **5-8** were performed at room temperature. Due to inconsistent mixing in the absence of stirring as a result of substrate insolubility, bioconversion plates were shaken at 750 rpm using a Heidolph Titramax 1000 plate shaker. 100 μM stock solutions were prepared for each enzyme. Substrate stock solutions were prepared as 100 mM solutions in DMSO, prepared fresh prior to use. From the 100 mM stocks, 5 μL of each substrate was added

to a polypropylene microtiter plate, and these were diluted with 85 µL of phosphate buffer (pH 7.5, 50 mM). To initiate the reactions, 10 µL of the 100 µM stock solution of each enzyme was added to the microtiter plate. Reactions were run in triplicate and allowed to react for one hour. To end the reactions, each reaction was quenched using one reaction volume of quench solution (200 µM 5-bromoindole as internal standard in methanol). These reactions were then further diluted 1:4 in water and processed the same as the general lysate bioconversions. Reactions were analyzed by UHPLC, using method C and the amount of product was quantified by using a calibration curve at 230 nm.

F. Kinetic characterization of WT-BS2 and 10D2 with 4a and 4b.

In general for kinetic characterization, 3x stock solutions of the enzyme were prepared (i.e. for 2.5 µM final concentration, stocks of 7.5 µM enzyme were prepared). Substrates were prepared as 1.5x stocks. 50 µL of the substrate stock solutions were added to a 96-well microtiter plate in triplicate, and the reactions were initiated by the addition of 25 µL of the enzyme stock solutions. Reactions were left at room temperature without any stirring until the timepoints were finished, at which point one volume of quench solution (200 µM internal standard in methanol, 100 µM final concentration) was added to the reactions. After the last timepoint was quenched, the reactions were processed as described for the general lysate bioconversions. Reactions were analyzed using UHPLC using method B. Abscisic acid was quantified using a calibration curve with the interal standard measured at 280 nm.

V. Synthesis

A. General procedure for the synthesis of IPTG esters 1a-1c.

To an oven-dried round bottomed flask under nitrogen atmosphere was added IPTG (500 mg, 2.09 mmol). The flask was evacuated and backfilled with nitrogen 3x before 3 mL 2,4,6-collidine was added to the flask. This solution was cooled to –35 °C in a 40:60 methanol:water bath containing dry ice. The corresponding acid chloride was added dropwise over 20 minutes. Upon addition of the acid chloride, the solution solidified into a white gummy solid. After 30 minutes, product was confirmed by TLC and the solution was removed from the dry ice bath and allowed to warm to room temperature. The resulting gummy solid was dissolved in DCM and purified by silica chromatography using a gradient of 1 column volume each of 4:1 DCM:Acetone, 3:1 DCM:Acetone, then 2:1 DCM:Acetone. Product was identified by staining TLC plates in $KMnO_4$, then collected and concentrated by rotary evaporation. Residual collidine was removed under high-vacuum.

6-O-Acetyl-IPTG (**1a**): Obtained 491 mg, 1.75 mmol. 84% Yield

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.43 – 4.35 (m, 2H), 4.30 (dd, *J* = 11.6, 6.9 Hz, 1H), 3.97 (q, *J* = 1.3 Hz, 1H), 3.74 (ddd, *J* = 7.0, 5.9, 1.2 Hz, 1H), 3.69 – 3.62 (m, 2H), 3.23 (hept, *J* = 6.8 Hz, 1H), 2.10 (s, 3H), 1.37 (dd, *J* = 9.9, 6.8 Hz, 6H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 171.05, 86.01, 75.90, 74.46, 70.52, 68.48, 62.93, 36.05, 24.23, 23.95, 20.83.

HRMS: calc. for $[C_{12}H_{22}O_6SNa]^+$ ([M+Na]$^+$: 303.0873, found 303.0873.

6-O-propionyl-IPTG (**1b**): Obtained 369 mg, 1.254 mmol. 60% Yield

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.42 – 4.32 (m, 2H), 4.29 – 4.20 (m, 1H), 3.92 (s, 1H), 3.69 (t, *J* = 6.5 Hz, 1H), 3.65 – 3.57 (m, 2H), 3.18 (hept, *J* = 6.8 Hz, 1H), 2.33 (q, *J* = 7.6 Hz, 2H), 1.32 (dd, *J* = 8.2, 6.8 Hz, 6H), 1.13 (t, *J* = 7.8 Hz, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 174.50, 85.95, 75.99, 74.49, 70.51, 68.54, 62.81, 36.00, 27.47, 24.22, 23.90, 9.03.

HRMS: calc. for $[C_{12}H_{22}O_6SNa]^+$ ([M+Na]$^+$: 317.1029, found 317.1030.

6-O-isobutyl-IPTG (**1c**): Obtained 290 mg, 0.94 mmol. 45 % Yield.

The procedure for **1c** is the same as **1a** and **1b**, but 0.1 equiv. 4-dimethylaminopyridine was added to the reaction before collidine.

^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.45 – 4.36 (m, 2H), 4.28 (dd, *J* = 11.5, 6.9 Hz, 1H), 3.95 (q, *J* = 1.2 Hz, 1H), 3.73 (ddd, *J* = 7.0, 5.9, 1.2 Hz, 1H), 3.70 – 3.61 (m, 2H), 3.23 (hept, *J* = 6.7 Hz, 1H), 2.59 (hept, *J* = 7.0 Hz, 1H), 1.36 (dd, *J* = 11.1, 6.8 Hz, 6H), 1.19 (d, *J* = 7.0 Hz, 6H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 177.20, 85.92, 76.03, 74.49, 70.55, 68.52, 62.75, 35.96, 33.94, 24.24, 23.88, 18.93, 18.89.

HRMS: calc. for $[C_{13}H_{24}O_6SNa]^+$ ([M+Na]$^+$: 331.1186, found 331.1187

6-O-trimethylacetyl-IPTG (**1d**): To an oven-dried round bottomed flask under nitrogen atmosphere was added IPTG (500 mg, 2.09 mmol), and 0.1 equiv. 4-dimethylaminopyridine (25 mg, 0.21

mmol). This was evacuated under vacuum and purged with nitrogen three times, after which 3 mL pyridine was added to the flask. The solution was cooled to 0 °C with an ice bath, at which point trimethylacetyl chloride (255 µL, 2.09 mmol) was added dropwise over 20 minutes. Upon addition of trimethylacetyl chloride the solution became a white gummy solid. After addition of trimethylacetyl chloride, the solution was allowed to stir for a half hour at which point the ice bath was removed and the gummy solid dissolved in DCM and purified via silica chromatography using a gradient of 1 column volume each of 4:1 DCM:Acetone, 3:1 DCM:Acetone, then 2:1 DCM:Acetone. Product was identified by staining TLC plates in KMnO4, then collected and concentrated under reduced pressure. Residual collidine was removed under high-vacuum until white crystals crashed out of solution.

Obtained 225 mg (1.46 mmol) 70 % yield.

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.36 – 4.27 (m, 2H), 4.17 (dd, *J* = 11.6, 7.0 Hz, 1H), 3.85 (s, 1H), 3.64 (t, *J* = 6.5 Hz, 1H), 3.57 (d, *J* = 8.3 Hz, 2H), 3.14 (p, *J* = 6.8 Hz, 1H), 1.27 (dd, *J* = 12.7, 6.7 Hz, 6H), 1.13 (s, 9H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 178.68, 85.86, 76.09, 74.51, 70.56, 68.58, 62.92, 38.80, 35.92, 27.14, 24.26, 23.83.

HRMS: calc. for $[C_{14}H_{26}O_6SNa]^+$ ([M+Na]$^+$: 345.1342, found 345.1344.

B. Synthesis of abscisic acid esters 4a-4d.

4a – methyl abscisic acid: To a stirring solution of ABA (250 mg, 0.94 mmol) in 5 mL of a 9:1 mixture of toluene to methanol was added a 2M solution of TMS-diazomethane in diethyl ether. The TMS-diazomethane solution was added dropwise until a yellow color persisted, at which point the solution was neutralized with glacial acetic acid until colorless. The solution was then concentrated under rotovap and washed with toluene until white crystals were obtained, and the compound was used without further purification. 261 mg obtained, quantitative yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (d, *J* = 16.1 Hz, 1H), 6.08 (d, *J* = 16.1 Hz, 1H), 5.88 (s, 1H), 5.69 (s, 1H), 3.64 (s, 3H), 2.47 – 2.17 (m, 2H), 1.94 (d, *J* = 1.3 Hz, 3H), 1.86 (d, *J* = 1.4 Hz, 3H), 1.05 (s, 3H), 0.95 (s, 3H).

^{13}C NMR (126 MHz, Methylene Chloride-d_2) δ 196.54, 165.54, 161.44, 148.88, 135.72, 127.24, 126.21, 117.15, 78.94, 50.24, 48.90, 40.65, 23.26, 22.06, 20.12, 17.90.

HRMS: calc. for $[C_{18}H_{26}O_4Na]^+$ ([M+Na]$^+$: 301.1410, found 301.1412.

4b – ethyl abscisic acid: To a stirring solution of ABA (250 mg, 0.94 mmol) in 5 mL DCM was added 1 equiv. of DBU (0.94 mmol, 140 µL, followed by 1.2 equiv. of bromoethane (1.13 mmol, 90 µL). This solution was allowed to stir overnight at room temperature. The next day, the solution was transferred to a separatory funnel and washed with saturated sodium bicarbonate, then brine and dried over magnesium sulfate. After concentrating the solution by rotary evaporation the resulting oil was taken up in DCM and the compound was purified via silica chromatography using a 6:1 hexanes:ethyl acetate solvent mixture. After removing solvent under reduced pressure, white crystals were obtained in 94% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, *J* = 16.1 Hz, 1H), 6.08 (d, *J* = 16.0 Hz, 1H), 5.87 (s, 1H), 5.68 (s, 1H), 4.10 (q, *J* = 7.1 Hz, 2H), 2.41 (d, *J* = 17.1 Hz, 1H), 2.28 – 2.17 (m, 1H), 1.94 (s, 3H), 1.85 (s, 3H), 1.22 (t, *J* = 7.1 Hz, 3H), 1.04 (s, 3H), 0.95 (s, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 197.64, 166.02, 162.31, 148.89, 136.05, 128.19, 127.08, 118.89, 79.66, 59.98, 49.78, 41.52, 24.32, 23.05, 21.15, 18.88, 14.30.

HRMS: calc. for $[C_{17}H_{24}O_4Na]^+$ ([M+Na]$^+$: 315.1567, found 315.1568

4c – isopropyl abscisic acid: ABA (250 mg, 0.94 mmol) was added to an oven-dried round bottomed flask that was evacuated and purged with nitrogen 3x. 1.5 equiv. N,N,N',N'-tetramethylazodicarboxamide (TMAD) (243 mg, 1.41 mmol) was added, followed by 2.5 mL anhydrous benzene. The solution was allowed to cool to 0 °C under nitrogen atomosphere in an ice bath and 1.5 equiv. tri-n-butylphosphine (350 µL, 1.41 mmol) was added, followed by 3 equiv. isopropanol (215 µL, 2.82 mmol). Upon addition of the alcohol, the solution solidified immediately. The ice bath was replaced with an oil bath and the solution heated to 60 °C at which point the mixture gradually went back into solution. The reaction was monitored by TLC until no further product formation was obtained, at which point the solution was concentrated under reduced pressure and the resulting residue taken up in DCM. A work up consisting of a 0.5 M HCl wash, followed by a brine wash was done and the solution was dried over magnesium sulfate. This was filtered away and the solution was dried under reduced pressure. After removing solvent, the resulting oil was purified via silica chromatography using a 6:1 hexanes:ethyl acetate solvent

mixture and concentrated under rotary evaporation until a white solid crashed out of solution. Obtained 201 mg (0.66 mmol), 70% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, *J* = 16.0 Hz, 1H), 6.06 (d, *J* = 16.1 Hz, 1H), 5.87 (s, 1H), 5.66 (s, 1H), 5.05 – 4.93 (m, 1H), 2.41 (d, *J* = 17.0 Hz, 1H), 2.22 (d, *J* = 17.2 Hz, 1H), 1.93 (d, *J* = 1.3 Hz, 3H), 1.86 (d, *J* = 1.4 Hz, 3H), 1.19 (dd, *J* = 6.2, 1.2 Hz, 6H), 1.04 (s, 3H), 0.95 (s, 3H).

^{13}C NMR (101 MHz, Methylene Chloride-d_2) δ 197.10, 165.35, 161.96, 148.64, 135.97, 128.04, 126.94, 119.08, 79.64, 67.06, 49.62, 41.34, 23.96, 22.74, 21.62, 20.80, 18.57.

HRMS: calc. for $[C_{18}H_{26}O_4Na]^+$ ([M+Na]$^+$: 329.1723, found 329.1725.

4d – t-butyl ABA: To a stirring solution of ABA (100 mg, 0.378 mmol) in N,N-dimethylacetamide (3 mL) was added benzyltriethylammonium chloride (86 mg, 0.378 mmol), followed by 26 equiv. Cs_2CO_3 (3.205 g, 9.86 mmol) and 48 equiv. 2-bromo-2-methylpropane (2.037 mL, 18.14 mmol). The mixture was placed into an oil bath and heated to 55 °C for 24 hours, at which point starting material could not be observed on TLC. The solution was diluted with DI water, and the product was extracted from the aqueous phase 3x with EtOAc. This solution was then washed with 0.5 M HCl, 5% LiCl, then brine. The organic layer was dried over magnesium sulfate and the solvent removed under rotary evaporation. The resulting residue was taken up in DCM and the product was purified via silica chromatography using a 3:1 hexanes:ethyl acetate solvent mixture. A clear oil was obtained, which after rinsing with diethyl ether and concentrating by rotary evaporation crashed out of solution as white crystals. Obtained 107.8 mg (0.34 mmol), 89% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 16.1 Hz, 1H), 6.03 (d, *J* = 16.1 Hz, 1H), 5.85 (s, 1H), 5.61 (s, 1H), 2.42 (d, *J* = 17.1 Hz, 1H), 2.23 (d, *J* = 17.1 Hz, 1H), 1.90 (d, *J* = 1.3 Hz, 3H), 1.86 (d, *J* = 1.4 Hz, 3H), 1.42 (s, 9H), 1.04 (s, 3H), 0.94 (s, 3H).

^{13}C NMR (126 MHz, Methylene Chloride-d_2) δ 196.56, 164.71, 161.59, 146.91, 134.96, 127.17, 126.09, 119.71, 79.30, 78.86, 48.94, 40.61, 27.21, 23.31, 22.07, 20.06, 17.87.

HRMS: calc. for $[C_{19}H_{28}O_4Na]^+$ ([M+Na]$^+$): 343.1880, found 343.1880.

C. General procedure for the synthesis of 3,3-dimethylacrylate esters (5-8).

To a stirring solution of 3,3-dimethylacrylic acid (500 mg, 5 mmol) in DCM was added 1.2 Equiv. DBU (6 mmol, 895 µL) and 1 equiv. of EDCI•HCl (5 mmol, 960 mg), followed by 1 equiv. DMAP (5 mmol, 610 mg). 1.5 equiv. of the corresponding alcohol was then added to the reaction mixture,

and the solution was allowed to stir overnight. The next day, the solution was worked up by washing the organic layer with 0.5 M HCl, followed by a solution of saturated bicarbonate and brine. The organic layer was then dried over Na_2SO_4, which was removed by filtering and the resulting organic layer was concentrated under reduced pressure. The resulting oil was then dissolved in a small amount of DCM and the product was purified by silica chromatography using 24:1 hexanes:ethyl actetate.

Characterization of compound **6**, cyclopropyl(phenyl)methyl 3-methylbut-2-enoate:
Yield: 483 mg (2.09 mmol), 43%, obtained as clear oil.
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 – 7.28 (m, 5H), 5.81 (s, 1H), 5.30 (d, *J* = 8.6 Hz, 1H), 2.19 (s, 3H), 1.93 (s, 3H), 1.43 – 1.27 (m, 1H), 0.69 – 0.53 (m, 3H), 0.48 – 0.39 (m, 1H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 165.99, 157.01, 140.97, 128.42, 127.75, 126.65, 116.33, 78.64, 27.49, 20.32, 16.81, 4.14, 3.20.
HRMS: calc. for $[C_{15}H_{18}O_2]^+$ ($[M]^+$): 230.1307, found 230.1306.

Characterization of compound **7**, cyclohexyl 3-methylbut-2-enoate:
Yield: 554 mg, (3.04 mmol), 61%, obtained as clear oil.
^{1}H NMR (400 MHz, $CDCl_3$) δ 5.66 (p, *J* = 1.4 Hz, 1H), 4.77 (td, *J* = 8.9, 4.2 Hz, 1H), 2.16 (d, *J* = 1.3 Hz, 3H), 1.80 – 1.68 (m, 3H), 1.66 – 1.49 (m, 3H), 1.49 – 1.28 (m, 5H), 1.27 (s, 2H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 166.13, 155.68, 116.62, 71.52, 31.71, 27.24, 25.37, 23.75, 20.04.
HRMS: calc. for $[C_{11}H_{18}O_2]^+$ ($[M]^+$): 182.1307, found 182.1305.

Characterization of compound **8**, 2-isopropyl-5-methylcyclohexyl 3-methylbut-2-enoate (menthylbutenoate):
Yield: 536 mg (2.25 mmol), 52%, obtained as pale yellow solid.
^{1}H NMR (400 MHz, $CDCl_3$) δ 5.64 (p, *J* = 1.4 Hz, 1H), 4.68 (td, *J* = 10.8, 4.4 Hz, 1H), 2.14 (d, *J* = 1.3 Hz, 3H), 2.04 – 1.95 (m, 1H), 1.91 – 1.81 (m, 4H), 1.65 (dp, *J* = 13.2, 3.1 Hz, 2H), 1.48 (dtq, *J* = 8.7, 6.2, 3.1 Hz, 1H), 1.36 (ddt, *J* = 14.1, 10.9, 3.1 Hz, 1H), 1.12 – 0.99 (m, 1H), 0.99 – 0.90 (m, 1H), 0.87 (dd, *J* = 6.8, 4.2 Hz, 7H), 0.74 (d, *J* = 7.0 Hz, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 166.35, 156.05, 116.56, 73.08, 47.16, 41.19, 34.36, 31.44, 27.38, 26.30, 23.57, 22.05, 20.78, 20.16, 16.44.

HRMS: calc. for $[C_{15}H_{26}O_2]^+$ ($[M]^+$): 238.1933, found 238.1932.

Safety statement.

No unexpected or unusually high safety hazards were encountered.

VI. Supplementary Tables and Figures

A. Table S1. List of plasmids used in this work.

Vector Name	Antibiotic resistance	RBS	Origin	Map*	Benchling Link
KJ106	carb	g10/strong RBS[6]	pSC101	a; acrylate biosensor	https://benchling.com/s/seq-MonsVwvPsM0AfmplSPcI
11-66	chlor	TTTAAGAA GGAGATAT ACAT[7]	cloDF13	b; IPTG biosensor	https://benchling.com/s/seq-KNkbS1fCHfh52E6cyB8k
KJ457	kan	TTTAAGAA GGAGATAT ACAT	pBR322	b; IPTG biosensor	https://benchling.com/s/seq-wWQSS1RtpJWWzkOSH8DJ
KJ478	chlor	TTTAAGAA GGAGATAT ACAT	cloDF13	b; IPTG biosensor	https://benchling.com/s/seq-R5g4rTU1ygkuxa8e13HB
KJ479	carb	TTTAAGAA GGAGATAT ACAT	p15A	b; IPTG biosensor	https://benchling.com/s/seq-SN6oiyRBxZ5Y47eSzemJ
KJ01	spec	sd8[8]	p15A	c; $RNAP_N$-LBD	https://benchling.com/s/seq-h8gdBuPwbGQpIgvf6W0L
KJ27	spec	SD8[8]	p15A	c; $RNAP_N$-LBD	https://benchling.com/s/seq-16GTSOz8gJKirsqmvSlV
KJ80	spec	sd8	p15A	c; MBP-$RNAP_N$-LBD	https://benchling.com/s/seq-KeDw4ktu0tOdmM7C7Qbh
KJ81	spec	sd8	p15A	c; MBP-$RNAP_N$-LBD	https://benchling.com/s/seq-iYaKpN6jUenJDUU4ny4K
KJ82	spec	sd8	p15A	c; MBP-$RNAP_N$-LBD	https://benchling.com/s/seq-0y6CE2HV3QG5xtfjXw3n
KJ86	carb	SD8/SD8	pSC101	c; RID-$RNAP_C$	https://benchling.com/s/seq-8ehftHP1Z7rmk5ldNCag
KJ87	kan	SD8/SD8	pBR322	c; RID-$RNAP_C$	https://benchling.com/s/seq-7DzoYusUuQ7dcAdobMSw
KJ83	carb	SD8/SD8	pSC101	c; MBP-RID-$RNAP_C$	https://benchling.com/s/seq-O1DCrEPAuivjDA14Skxu
KJ84	carb	SD8/SD8	pSC101	c; MBP-RID-$RNAP_C$	https://benchling.com/s/seq-5c4fQLq1CsewrYjIosft
KJ85	carb	SD8/SD8	pSC101	c; MBP-RID-$RNAP_C$	https://benchling.com/s/seq-F73j5yPVD9XRKFBw8rsN
8-15	spec	sd8	p15A	d; $RNAP_N$-ABI	https://benchling.com/s/seq-AIPz2A0jbaUojmG7pY7u
KJ77	spec	SD4[8]	p15A	d; $RNAP_N$-ABI	https://benchling.com/s/seq-gHkHeiEhMM4oWrHhu3sY
KJ78	spec	SD8	p15A	d; $RNAP_N$-ABI	https://benchling.com/s/seq-ZNA3Wf5Palnc6SEVvQy7
KJ71	carb	SD8/sd8	pSC101	d; PYL-$RNAP_C$	https://benchling.com/s/seq-4UXHxwEGhWZKuijxsW3O
KJ72	carb	SD8/SD4	pSC101	d; PYL-$RNAP_C$	https://benchling.com/s/seq-6nLhyNLiYX75FVj7nnxE

KJ73	carb	SD8/SD8	pSC101	d; PYL-$RNAP_C$	https://benchling.com/s/seq-rsiKrP1zCFb0Aqv4NwkO
KJ74	kan	SD8/sd8	pBR322	d; PYL-$RNAP_C$	https://benchling.com/s/seq-5d7mWAsF4wlPKFH1139i
KJ75	kan	SD8/SD4	pBR322	d; PYL-$RNAP_C$	https://benchling.com/s/seq-lJFj27WGEaJ7Tdat7quf
KJ76	kan	SD8/SD8	pBR322	d; PYL-$RNAP_C$	https://benchling.com/s/seq-JL0mmpXjHh2zUIhaWEhk
KJ123	carb	SD8/SD8	pUC19	d; PYL-$RNAP_C$	https://benchling.com/s/seq-sZMjEwlB97A8mOJsdIAP
KJ223	kan	SD8/SD8	pBR322	d; PYL-$RNAP_C$	https://benchling.com/s/seq-208A3o2Ib9sdWOMAvu6L
KJ89	chor	AATGACCGCT	cloDF13	e; BS2 expression	https://benchling.com/s/seq-PJGygHC1xGI1N132mBBm
KJ122	kan	GAAGGAG	pBR322	e; BS2 expression	https://benchling.com/s/seq-Cd1UfTOPGd4vJN4TMYjc

*vector maps for each construct type shown in Supplementary Figure 1.

B. Table S2. Sequences of top 8 variants from PACS compilation plate screen.

Identity	**Plate**	**Mutations**	**Normalized lysate conversion**	**New Mutations**	**Non-silent mutations**
P10-D-02	P10	E18G, M193T, M221T, K342E, E352G, T462A, N473S	456	E18G, M193T, M221T, E352G, T462, N473S	E18G, M193T, M221T, E352G, T462, N473S
P05-F-05	P05	L68P, K342E, E424G	356	L68P, E424G	L68P
P01-B-08	P01	N240S, A291A, K342E	251	N240S, A291A	N240S
P02-G-04	P02	F39L, N240S, A291A, K342E	233	F39L, N240S, A291A	F39L, N240S
P08-F-11	P08	L67L, P76R, E171V, T220A, D260D, D320G, K342E	215	L67L, P76R, T220A, D260D, D320G	P76R, T220A, D320G
P02-E-06	P02	L334F, M357L, R365C	203	L334F, M357L, R365C	L334F, M357L, R365C
P08-E-06	P08	K14R, Q36Q, R263W, F405L	199	K14R, Q36Q, R263W, F405L	K14R, R263W, F405L
P07-A-07	P07	P90T, I130V, A203A, A227A, N269S, L339P, K479K	181	P90T, I130V, A203A, A227A, N269S, L339P	P90T, I130V, N269S, L339P

Table S3. Kinetic parameters for WT-BS2 and evolved variant 10D2 on substrates 4a & 4b.

	4a			4b		
Variant	K_{cat} (min^{-1})	K_M (μM)	K_{cat}/K_M (min^{-1} μM^{-1})	K_{cat} (min^{-1})	K_M (μM)	K_{cat}/K_M (min^{-1} μM^{-1})
WT	0.092	111	0.000829	0.06784	143.2	0.000474
10D2	1.583	5615	0.00282	3.244	1265	0.00256

Table S4. Methods for UHPLC and UHPLC-MS analysis

Method A:

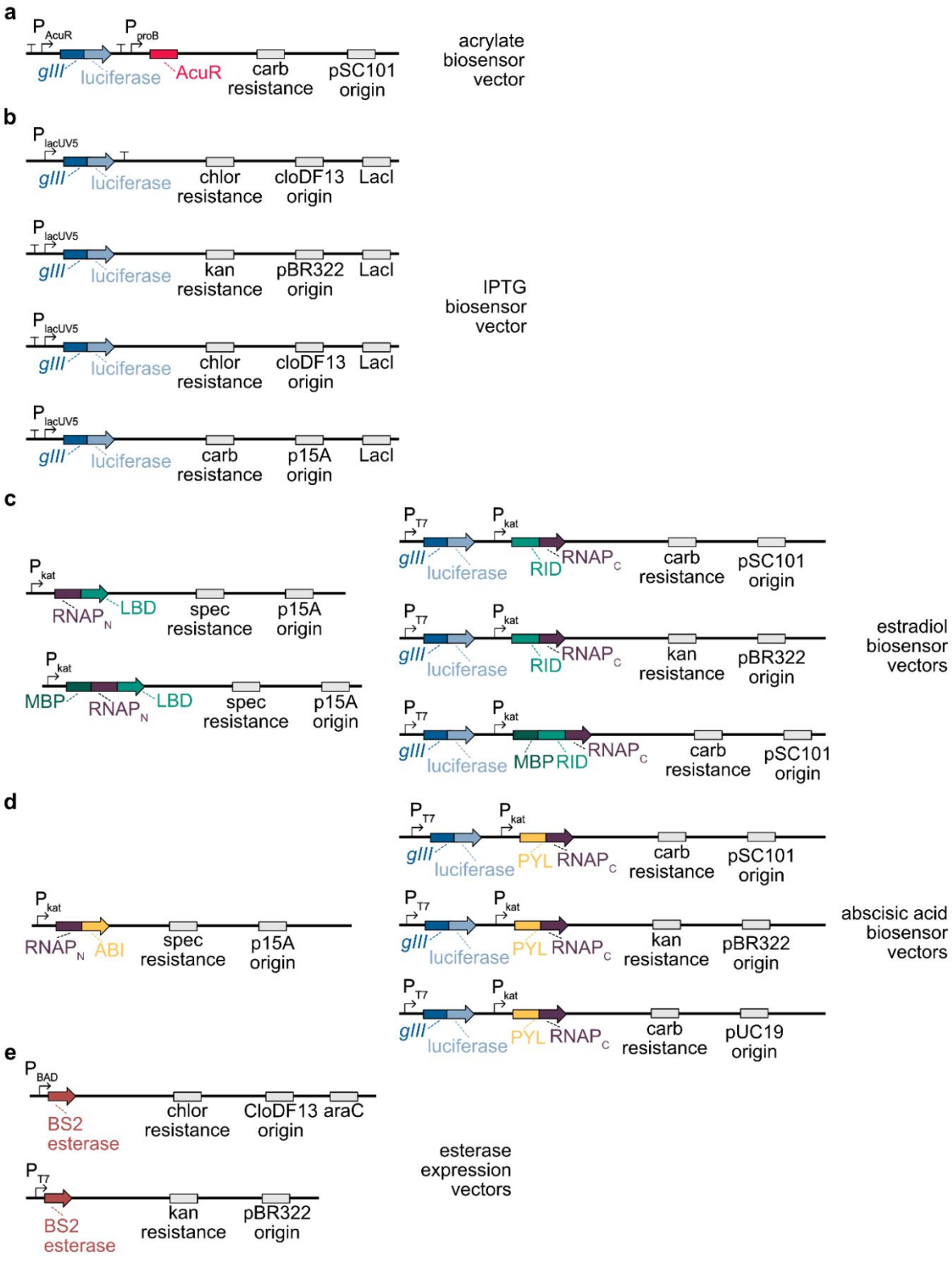
UHPLC-MS ABA	Time (min)	Starting %B	Ending %B
Mobile Phase A = Water + 0.1 % FA	0-0.5	30	30
Mobile Phase B = Acetonitrile + 0.1 % FA	0.51-1.25	30	50
	1.26-1.3	50	80
	1.31-1.75	80	95
	1.76-2.25	95	95
	2.26-2.5	30	30
	0.5 post run	30	30

Method B:

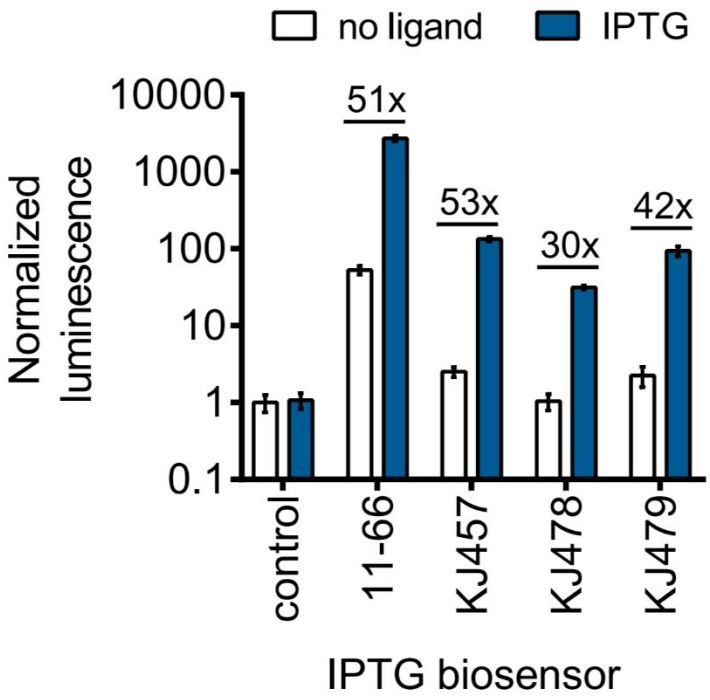
UHPLC-ABA	Time (min)	Starting %B	Ending %B
Mobile Phase A = Water + 0.1 % TFA	0-0.5	25	25
Mobile Phase B = Acetonitrile + 0.1 % TFA	0.51-1.2	25	45
	1.21-2	60	68
	2.01-2.5	68	95
	2.51-2.8	95	95
	2.81-3	25	25
	0.5 post run	25	25

Method C:

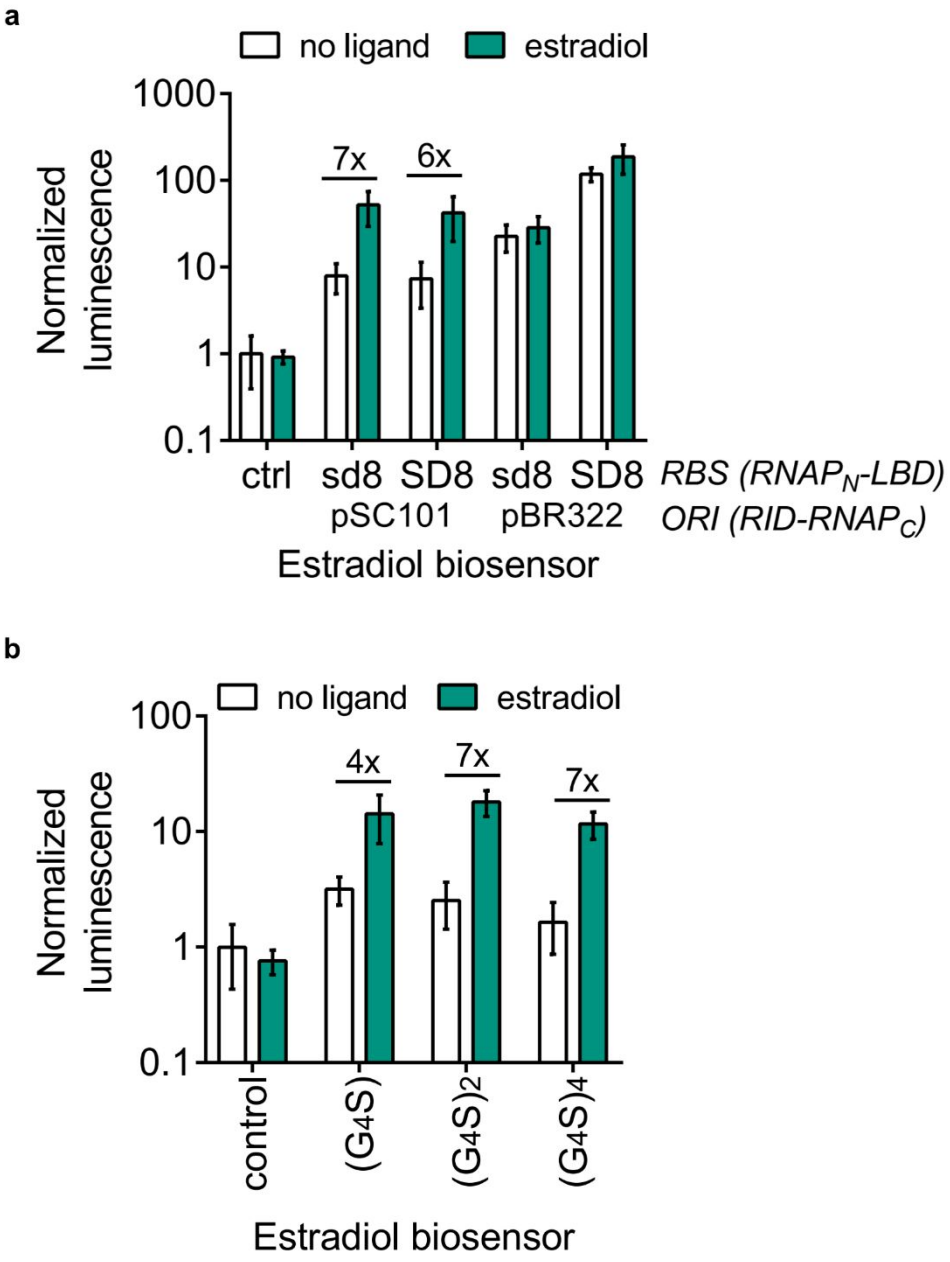
UHPLC - DMA Esters	Time (min)	Starting %B	Ending %B
Mobile Phase A = Water + 0.1 % TFA	0-0.5	25	25
Mobile Phase B = Acetonitrile + 0.1 % TFA	0.51-2	25	50
	2.01-3.5	50	95
	3.51-4.5	95	95
	4.51-5	25	25
	1.0 post run	25	25



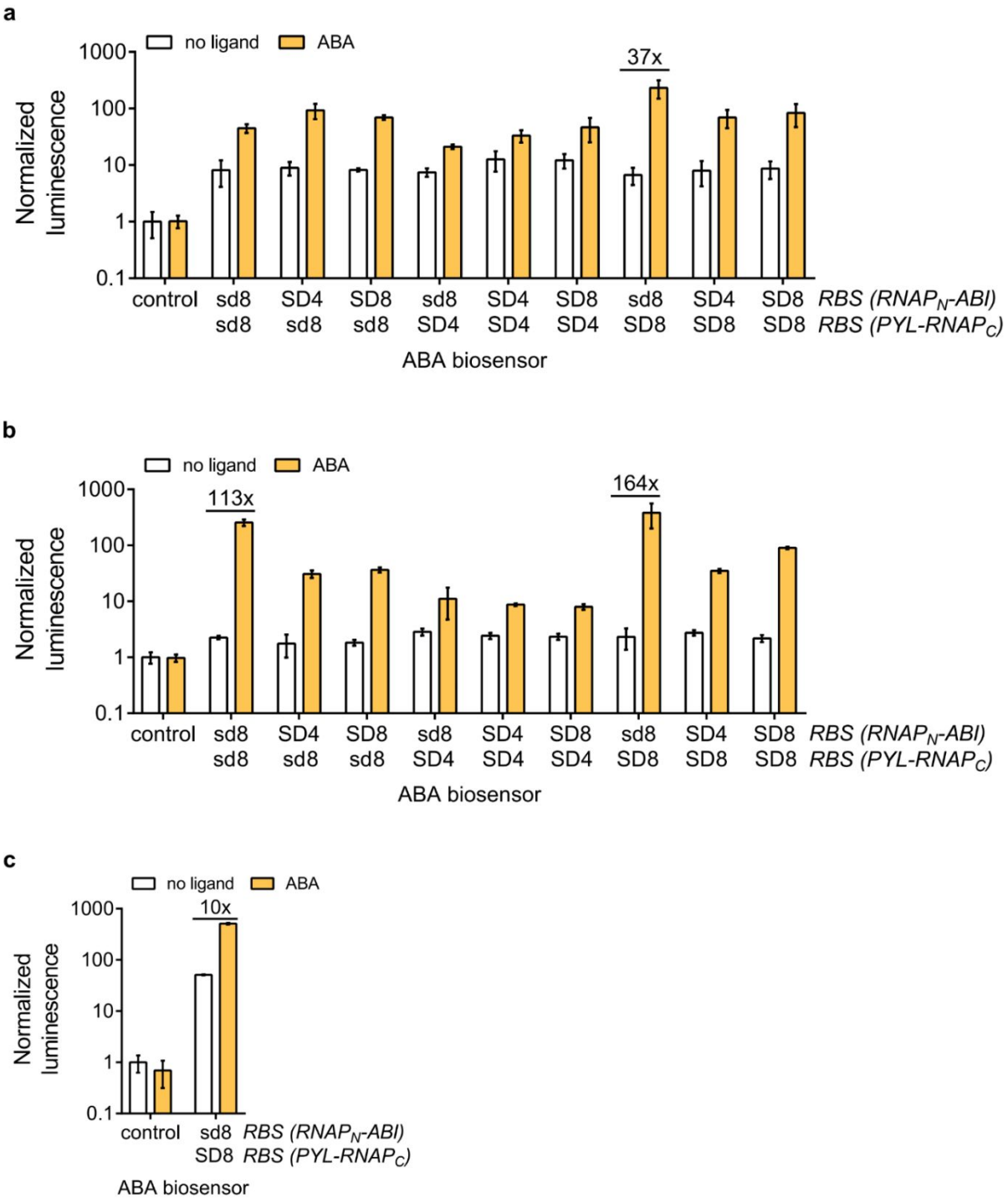
C. Figure S1. Vector maps for constructs used in this work. (a-e) Vector maps corresponding to vectors listed in Supplementary Table 1.



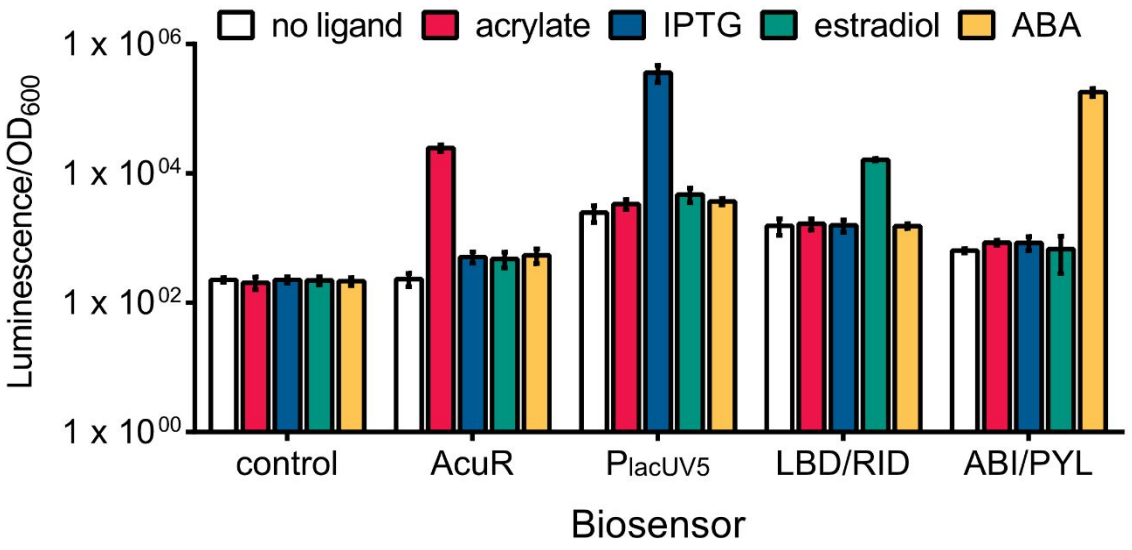
D. Figure S2. IPTG biosensor optimization. *E. coli* expressing IPTG biosensor for terminator and origin optimization (shown in Table S1 and Figure S1) were incubated in the absence or presence of IPTG (500 μM) for 3 h and then analyzed for luminescence. Error bars are the standard deviation of n = 4 replicates.



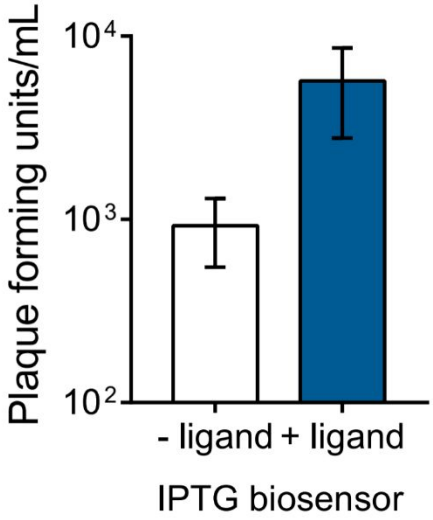
E. Figure S3. Estradiol biosensor optimization. *E. coli* expressing estradiol biosensor for (a) RBS and origin optimization (shown in Table S1 and Figure S1) or (b) improved solubility via MBP-(G_4S) linker fusions were incubated in the absence or presence of estradiol (250 μM) for 3 h and then analyzed for luminescence. Error bars are the standard deviation of n = 4 replicates.



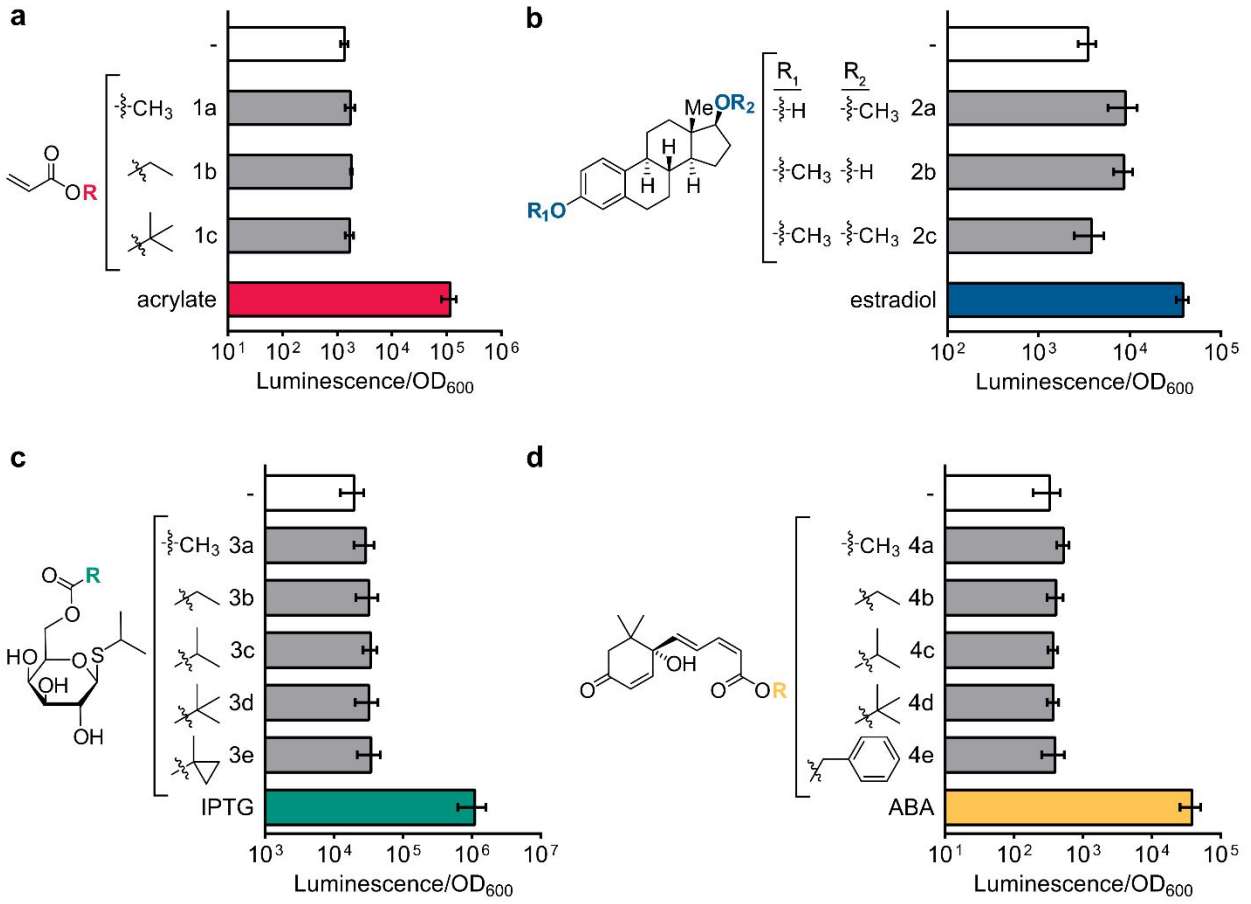
F. Figure S4. ABA biosensor optimization. *E. coli* expressing ABA biosensor for RBS and origin optimization with (a) psC101 (b) pBR322, or (c) pUC19 for PYL-RNAP$_C$ (shown in Table S1 and Figure S1) were incubated in the absence or presence of ABA (250 μM) for 3 h and then analyzed for luminescence. Error bars are the standard deviation of n = 4 replicates.



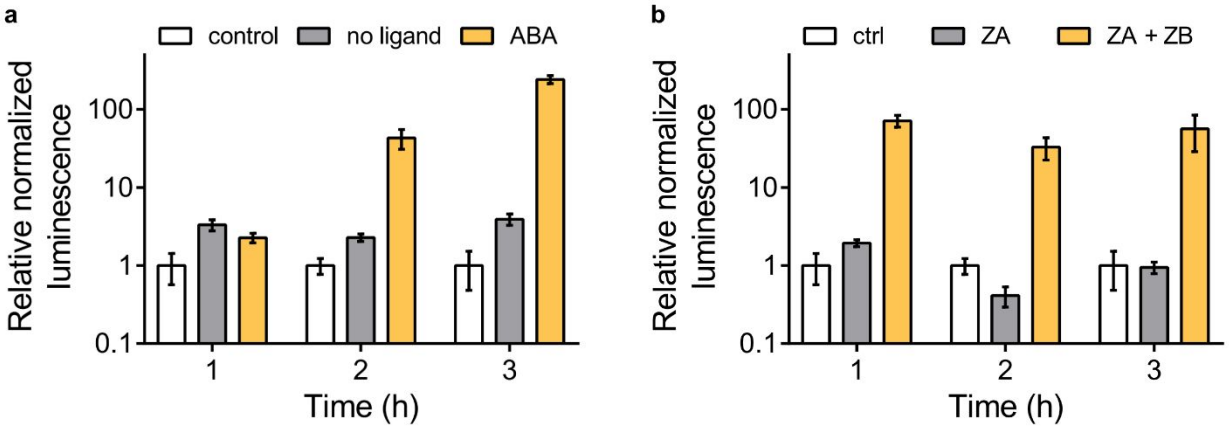
G. Figure S5. Development of biosensors for small molecule detection. Non-normalized luminescence data from Figure 1b. *E. coli* expressing plasmids shown in Figure 1a were incubated in the absence or presence of small molecule for 3 h and then analyzed for luminescence. Error bars are the standard deviation of n = 4 replicates.



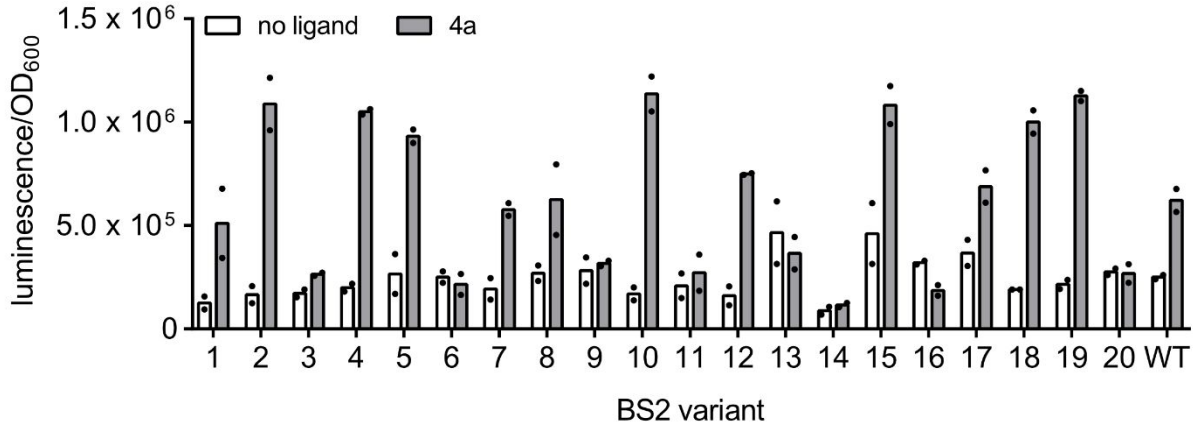
H. Figure S6. Phage replication of IPTG biosensor. Phage cultures from Figure 1c were diluted less (1:10) prior to analysis for phage replication. Error bars are the standard deviation of n = 4 replicates.



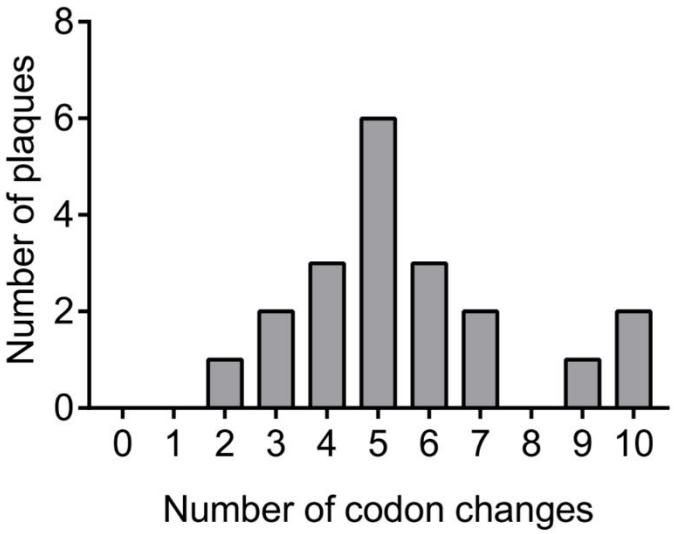
I. Figure S7. Small molecule biosensors can be used to detect selective biocatalysis. Non-normalized luminescence data from Figure 2. (a) *E. coli* expressing AcuR biosensor were incubated in the absence or presence of acrylate esters or acrylate for 3 h and then analyzed for luminescence. (b) *E. coli* expressing estradiol biosensor were incubated in the absence or presence of methylated estradiol or estradiol for 3 h and then analyzed for luminescence. (c) *E. coli* expressing IPTG biosensor were incubated in the absence or presence of IPTG esters or IPTG for 3 h and then analyzed for luminescence. (d) *E. coli* expressing ABA biosensor were incubated in the absence or presence of ABA esters or ABA for 3 h and then analyzed for luminescence. Error bars are the standard deviation for n = 4 replicates.



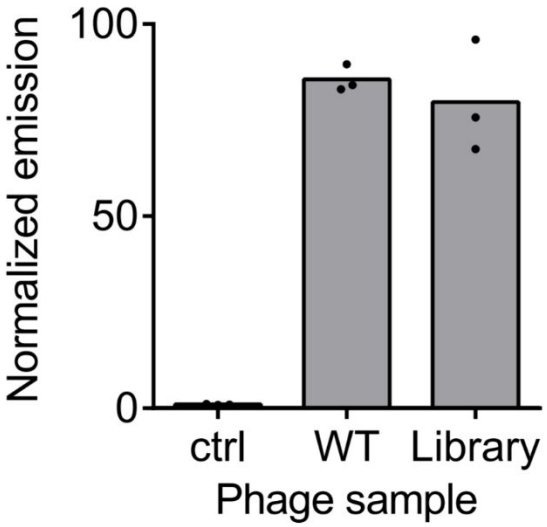
J. Figure S8. ABA incubation time prior to biosensor expression. *E. coli* expressing (a) ABA biosensor or (b) a non-small molecule dependent biosensor composed of interacting zipper peptides fused to split T7 RNAP ($RNAP_N$-ZA and ZB-$RNAP_C$) were incubated for various times (1-3 h) with arabinose (10 μM) to induce expression of $RNAP_N$-ABI or $RNAP_N$-ZA. After arabinose induction of the ABA biosensor, the cultures were incubated in the absence or presence of ABA (250 μM) for 3 h and then analyzed for luminescence. After arabinose induction of the zipper peptide biosensor, the cultures were incubated for 2 h and then analyzed for luminescence. Error bars are the standard deviation of n = 4 replicates.



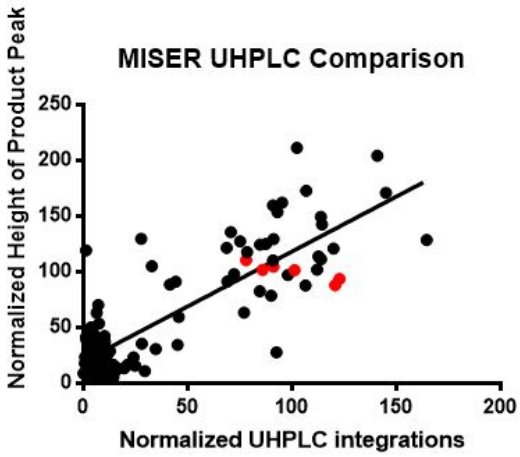
K. Figure S9. Screening of BS2 variants after PACE. Lagoon samples of BS2 phage were sub-cloned into an arabinose-inducible construct and assayed in *E. coli* expressing the ABA biosensor with no ligand or 4a for luciferase output.



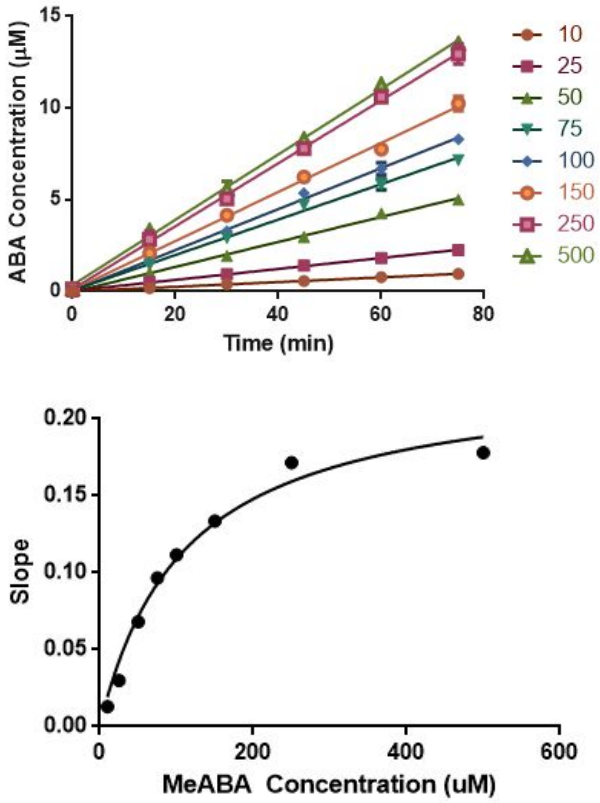
L. Figure S10. Library of BS2 phage variants generated via error prone PCR. Codon changes per gene for 20 randomly selected plaques from BS2 phage library.



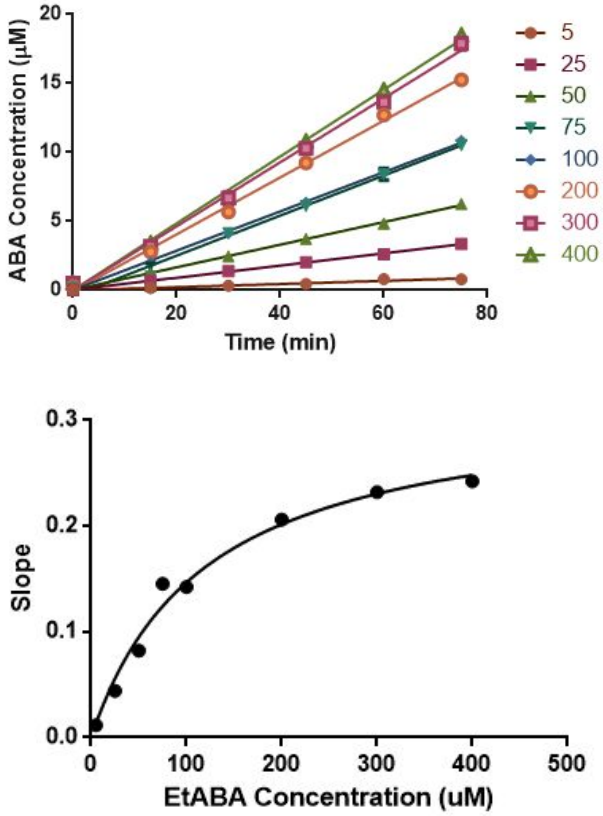
M. Figure S11. BS2 phage library contains active enzyme variants. Control phage (HRV), BS2 WT, or BS2 phage library (1 µL) was incubated with a fluorescein α-cyclopropyl ester (fluorescein-CM_2) fluorogenic molecule and 1059 cells overnight. Cultures were then analyzed for emission. Three samples of bulk phage (1 x 10^{12} PFU/mL) were analyzed.



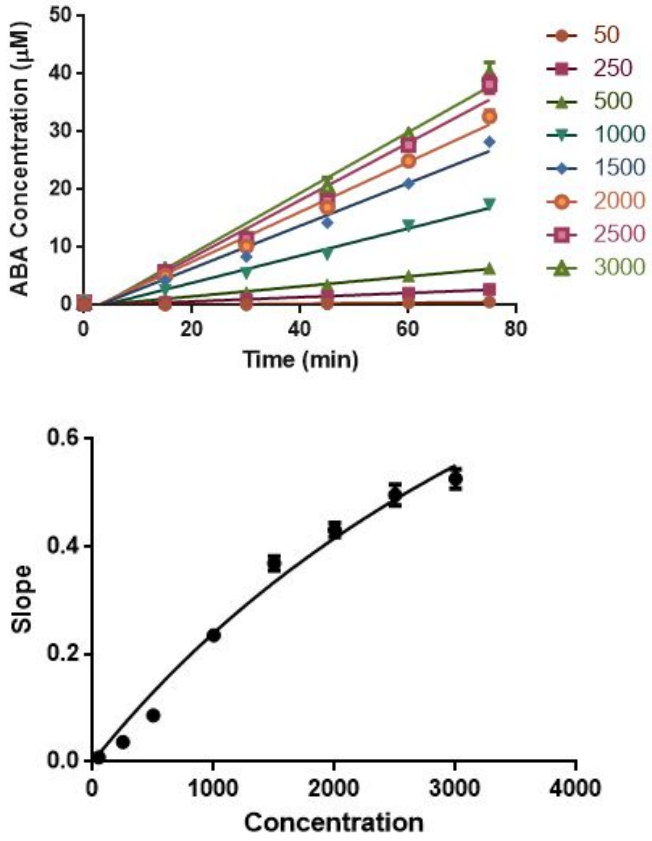
N. Figure S12. Low-throughput UHPLC compared to high-throughput MISER screen, with WT conversion normalized to 100 for both. WT values are highlighted in red. Pearson correlation coefficient was calculated to be 0.7234.



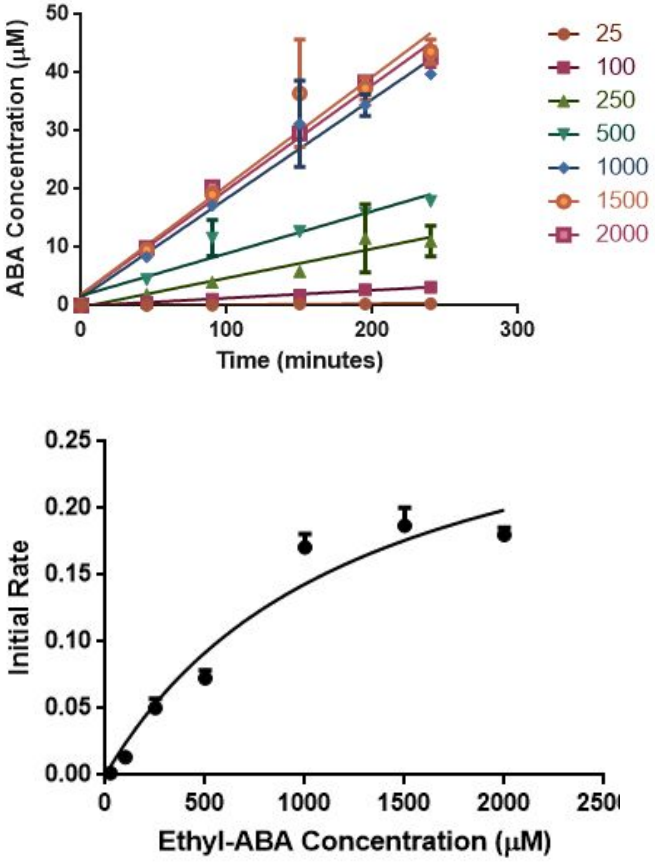
O. Figure S13. WT-BS2 kinetics with substrate 4a, methyl abscisic acid. Final enzyme concentration was 2.5 µM, with timepoints taken every 10 minutes. (a) Conversion vs time (b) Michaelis-Menten plot used to calculate K_M, K_{cat}. Slope calculations and kinetic parameters were calculated using Graphpad Prism.



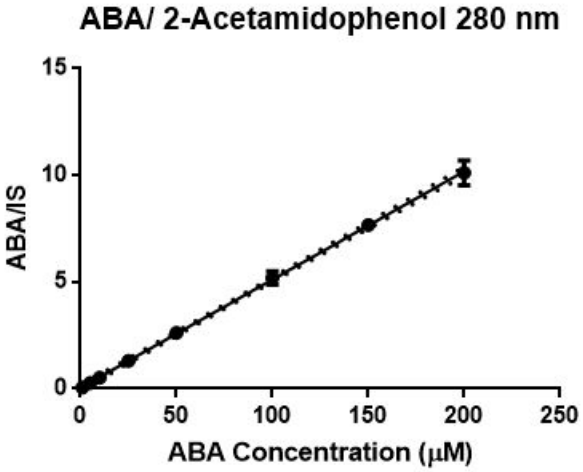
P. Figure S14. WT-BS2 kinetics with substrate 4b, ethyl abscisic acid. Final enzyme concentration was 5 µM, with timepoints taken every 10 minutes. (a) Conversion vs time (b) Michaelis-Menten plot used to calculate KM, Kcat. Slope calculations and kinetic parameters were calculated using Graphpad Prism.



Q. Figure S15. 10D2 kinetics with substrate 4a, methyl abscisic acid. Final enzyme concentration was 1 µM, with timepoints taken every 10 minutes. (a) Conversion vs time (b) Michaelis-Menten plot used to calculate KM, Kcat. Slope calculations and kinetic parameters were calculated using Graphpad Prism.



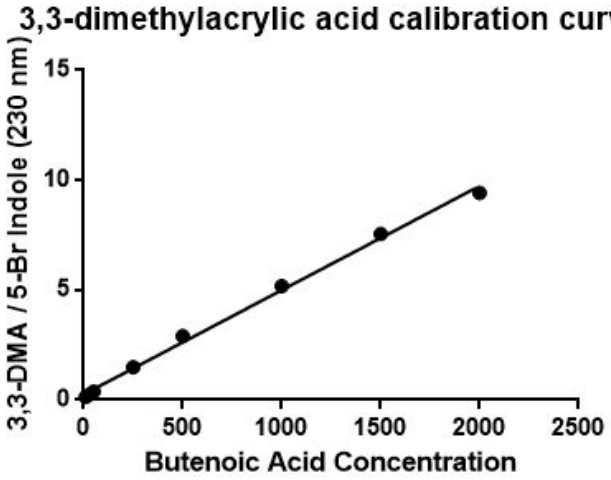
R. Figure S16. 10D2 kinetics with substrate 4a, methyl abscisic acid. Final enzyme concentration was 100 nM, with timepoints taken every 10 minutes. (a) Conversion vs time (b) Michaelis-Menten plot used to calculate KM, Kcat. Slope calculations and kinetic parameters were calculated using Graphpad Prism.



Goodness of Fit	
R square	0.9964

Equation	Y = 0.05072*X + 0.03598

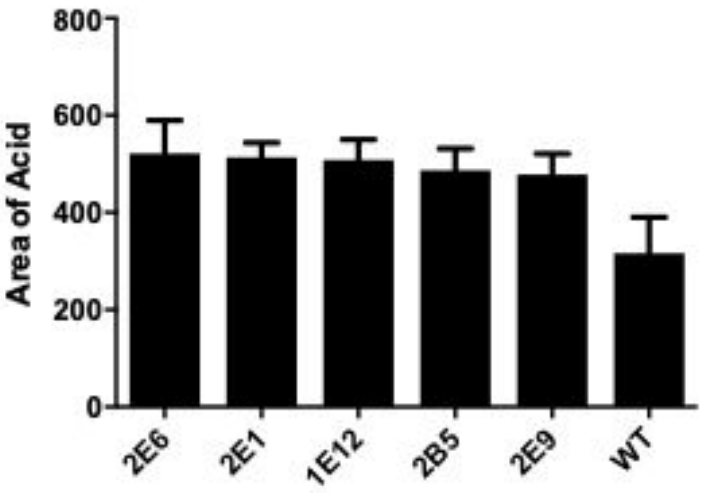
S. Figure S17. Calibration curve for abscisic acid with 2-acetamidophenol as the internal standard.



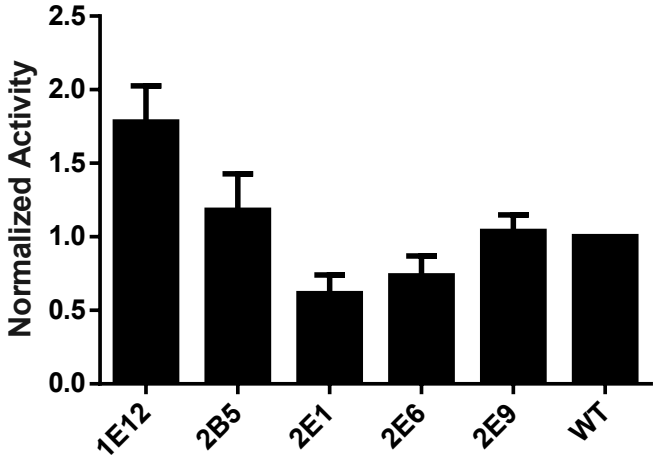
Goodness of Fit	
R square	0.9960

Equation	Y = 0.004742*X + 0.2518

T. Figure S18. Calibration curve for 3,3-dimethylacrylic acid with 5-bromo indole as the internal standard.



A. Figure S19. Lysate screening of the compilation plate for the hydrolysis of substrate **5.** Area of the 3,3-dimethylacrylic acid was used to determine the most active variants. Results are the average of two measurements.



A. Figure S20. Hydrolysis of substrate **5**. 10 μM of BS2 variant was used to hydrolyze 5 mM of substrate for one hour. Each bar represents the average of three measurements.

VII. NMR Spectra

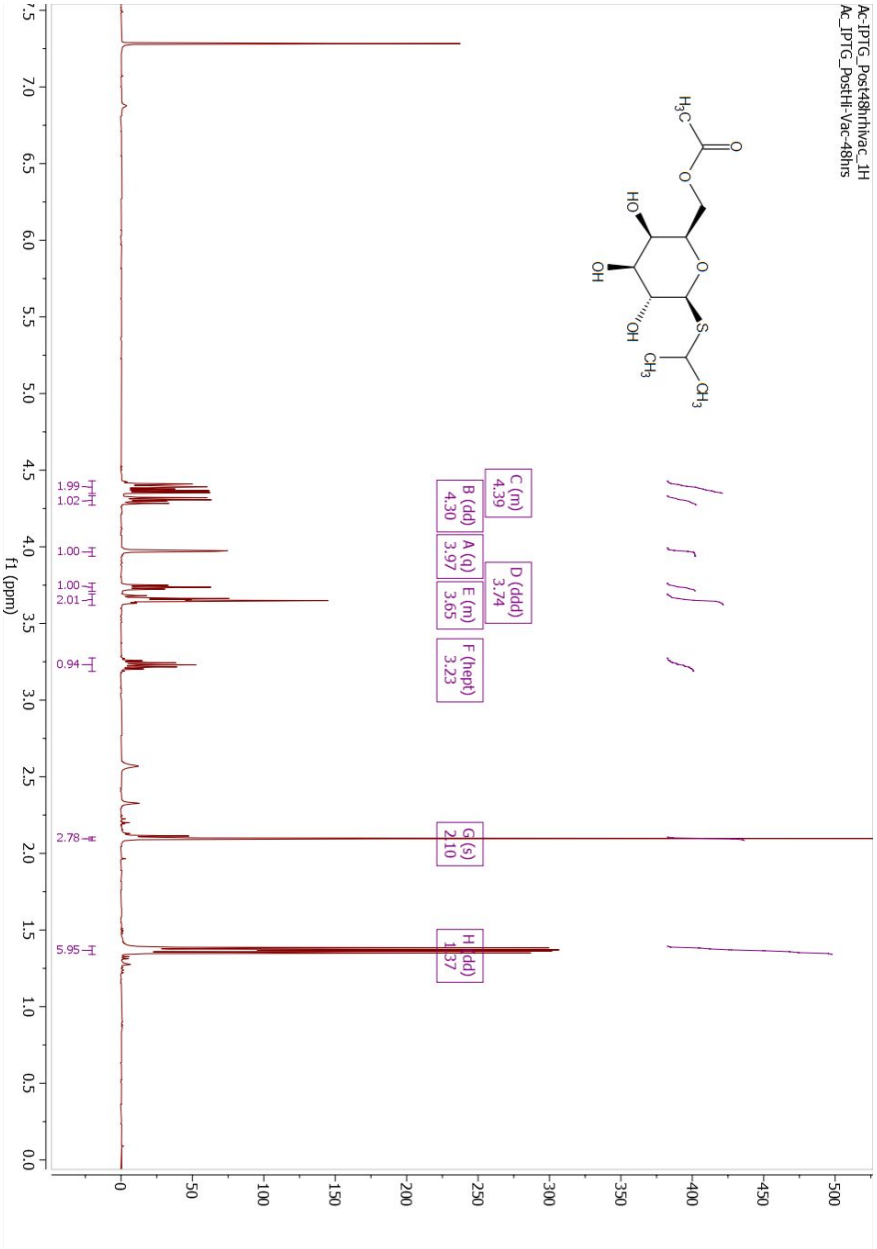


Figure S21: ^{1}H NMR of substrate 1a

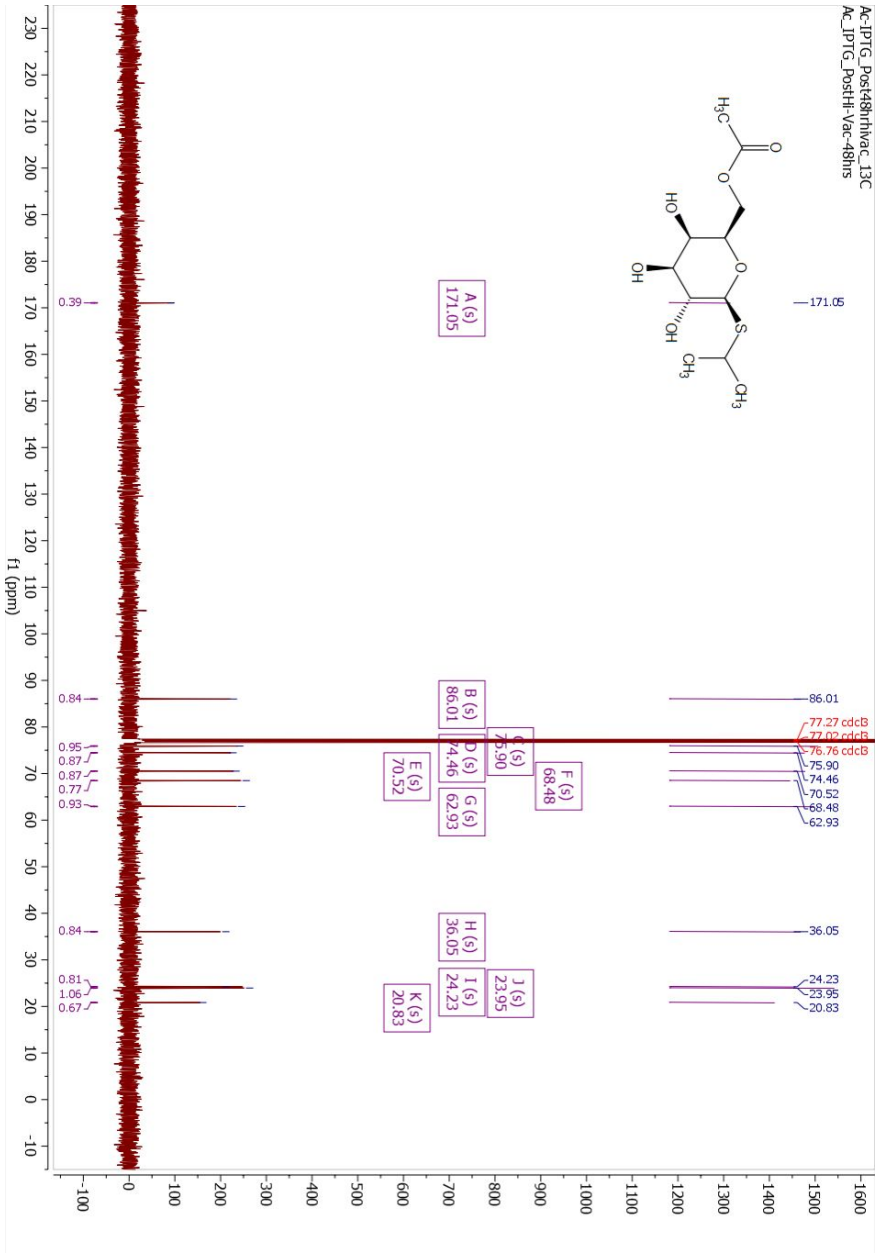


Figure S22: ^{13}C NMR of substrate 1a

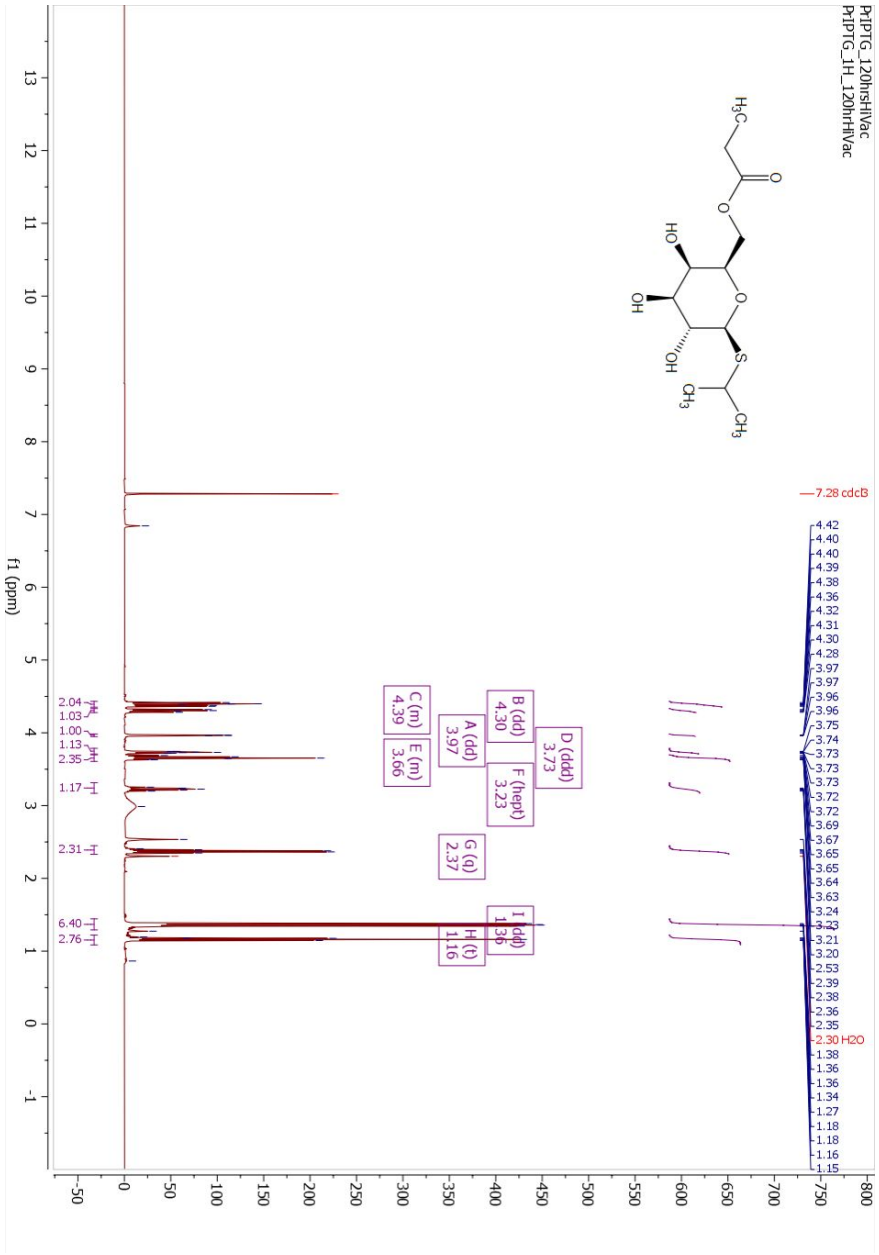


Figure S23: ^{1}H NMR of substrate 1b

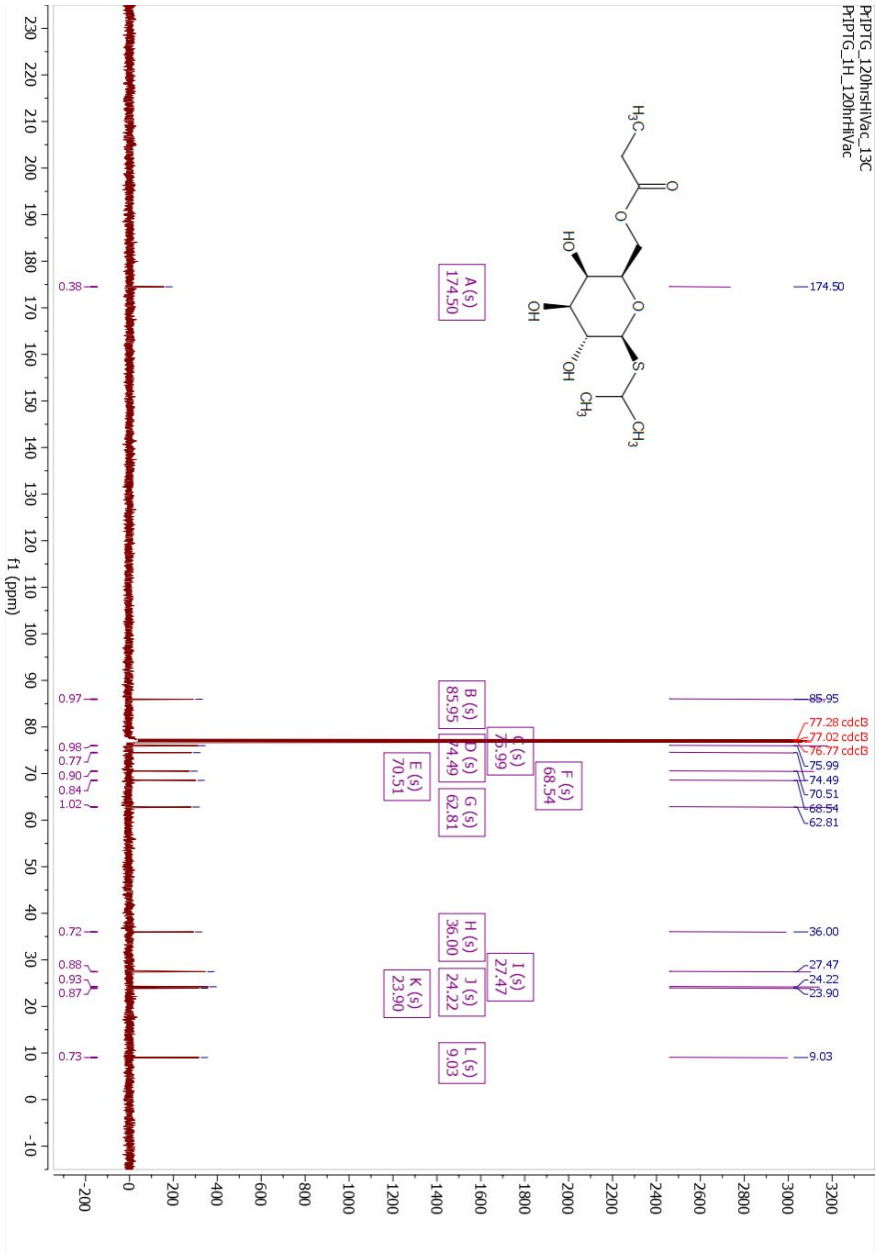


Figure S24: ^{13}C NMR of substrate 1b

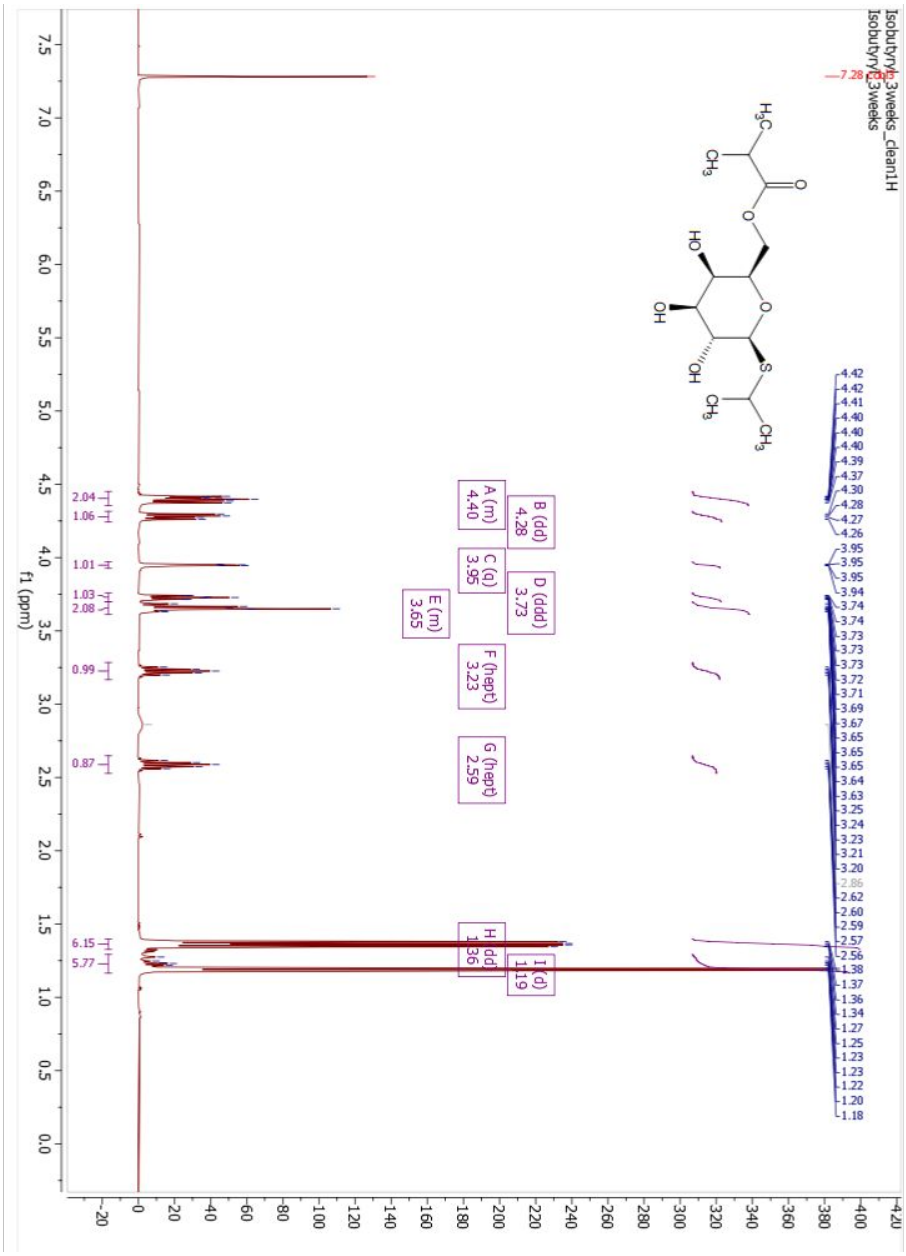


Figure S25: ^{1}H NMR of substrate 1c

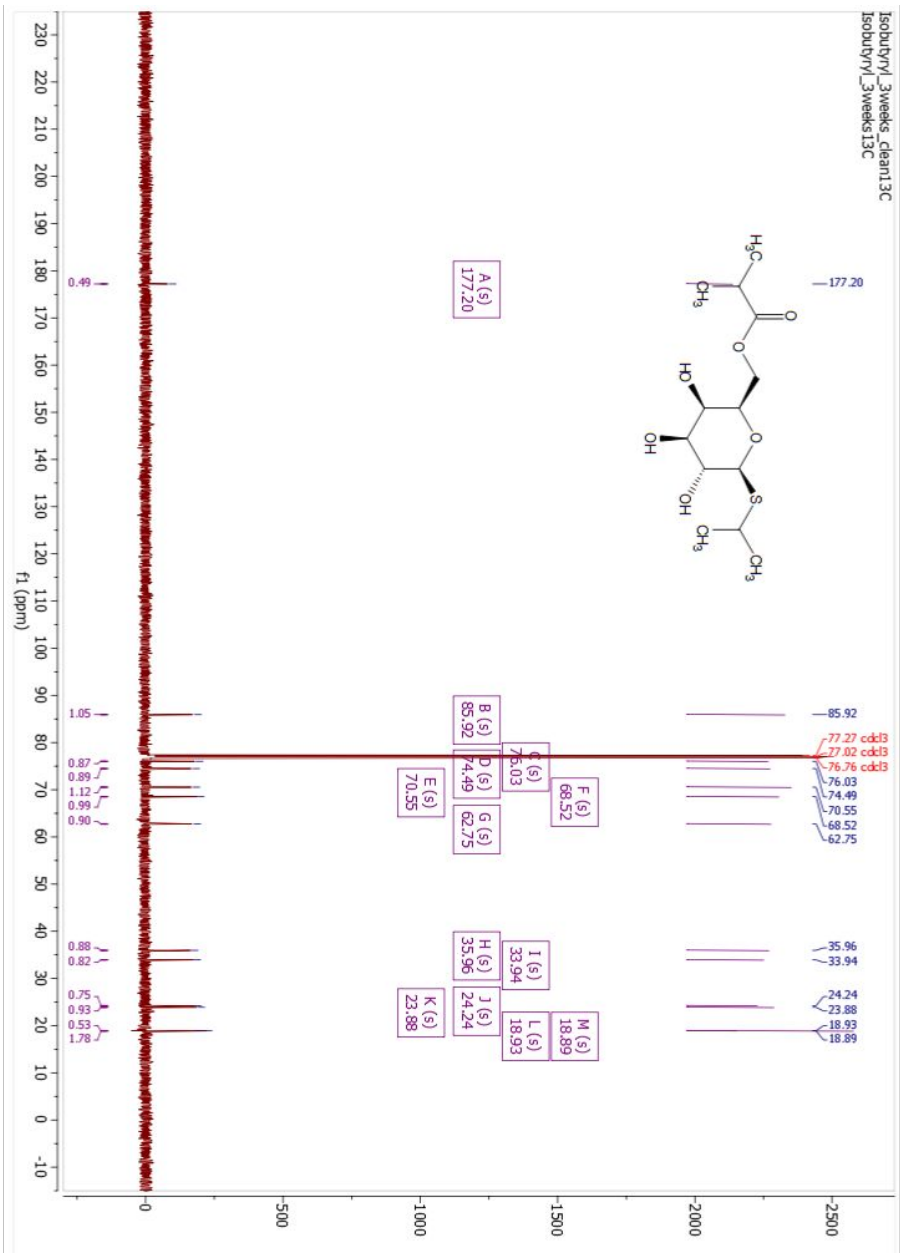


Figure S26: ^{13}C NMR of substrate 1c

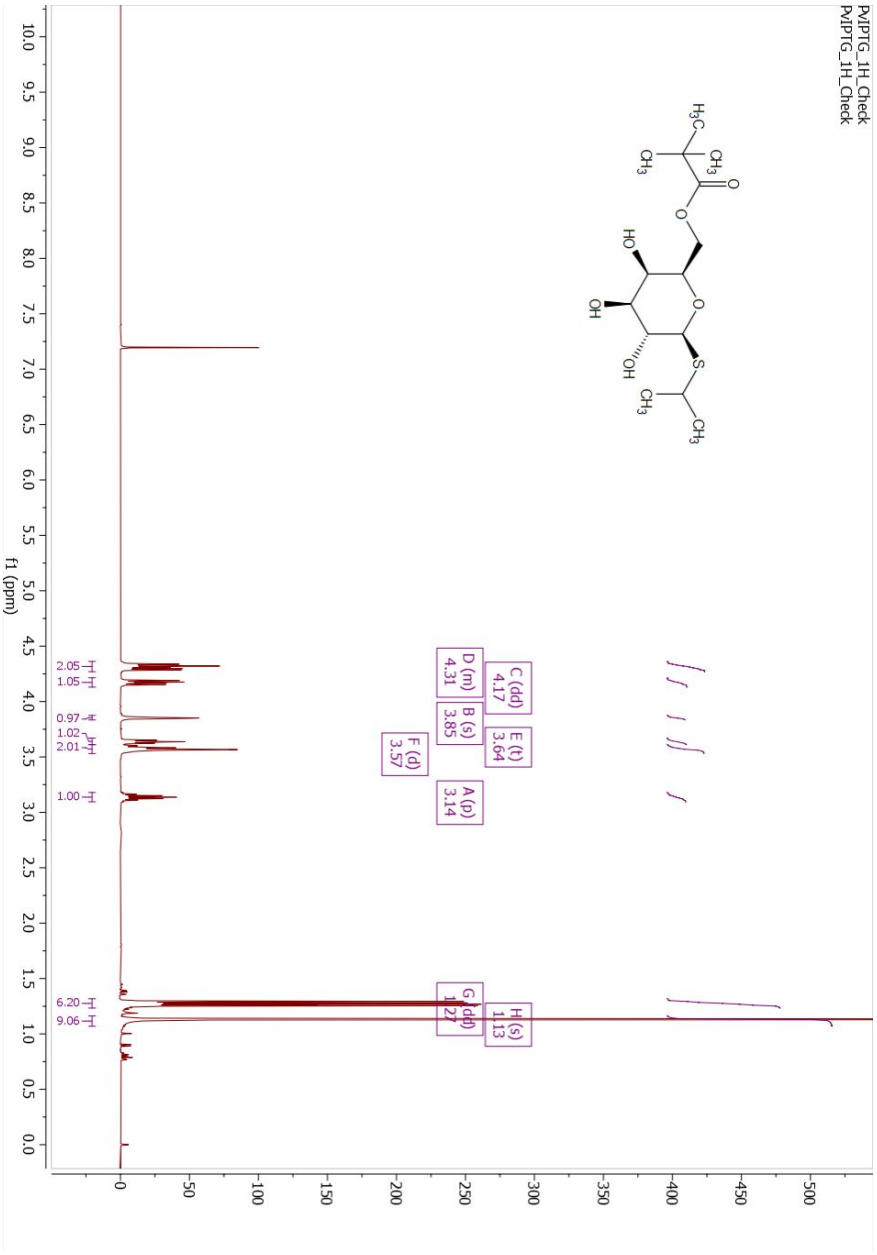


Figure S27: ^{1}H NMR of substrate 1d

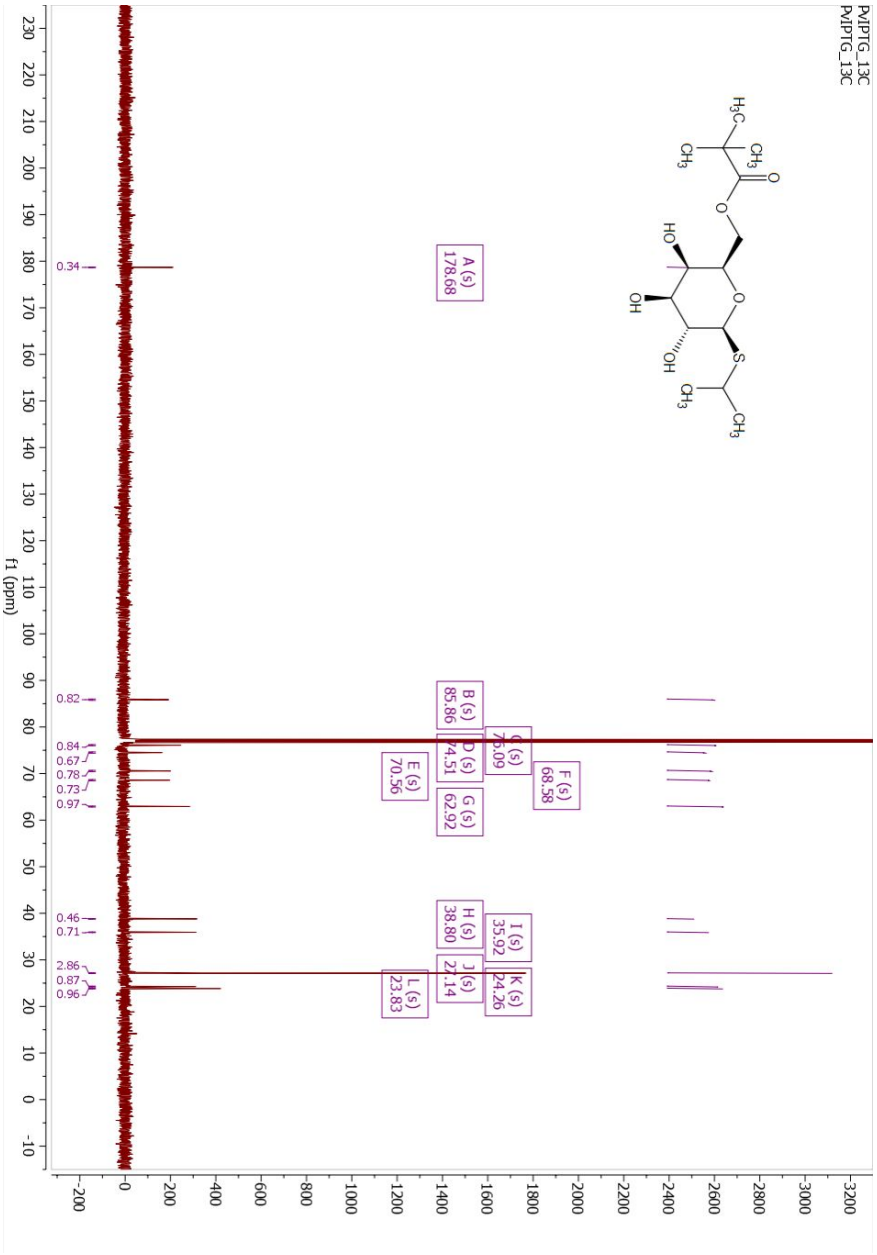
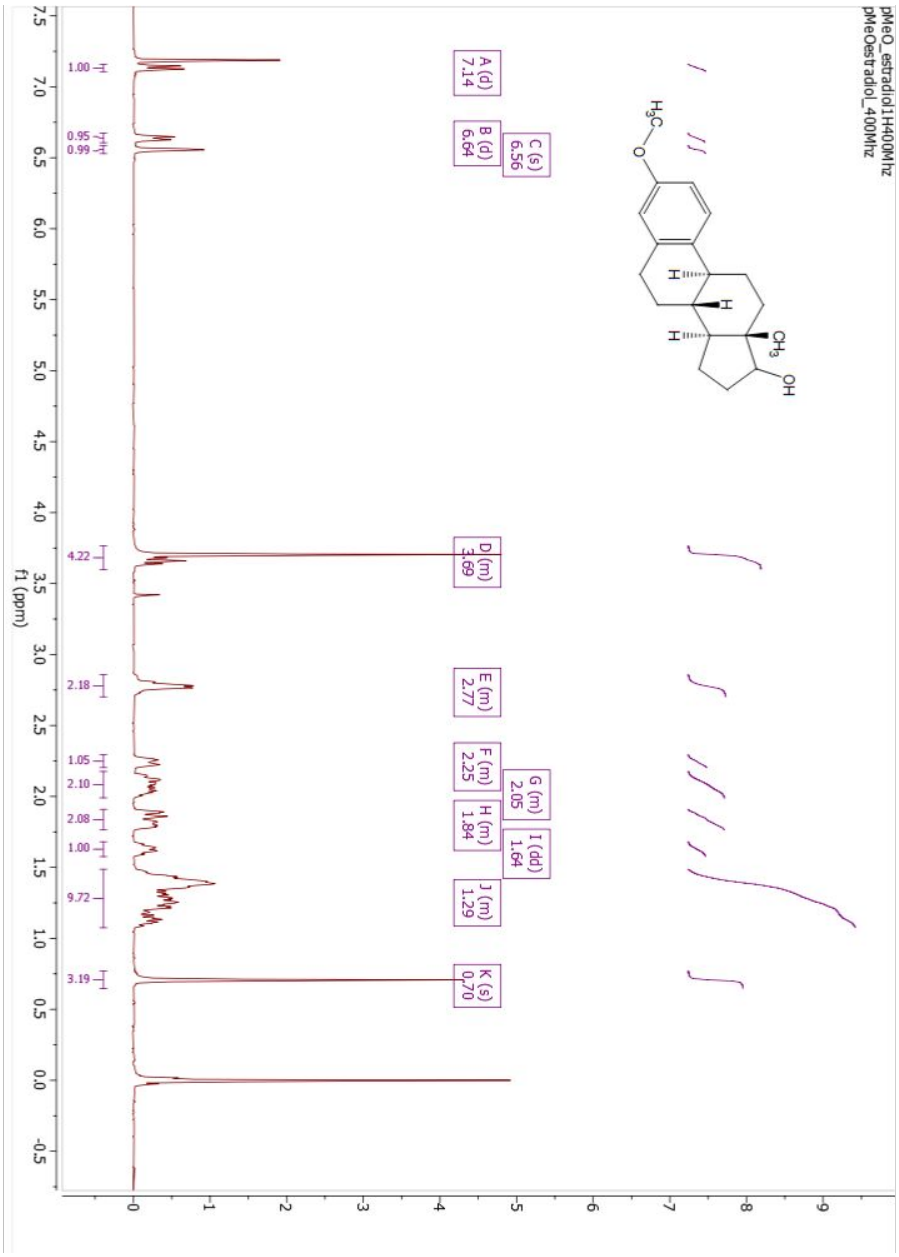
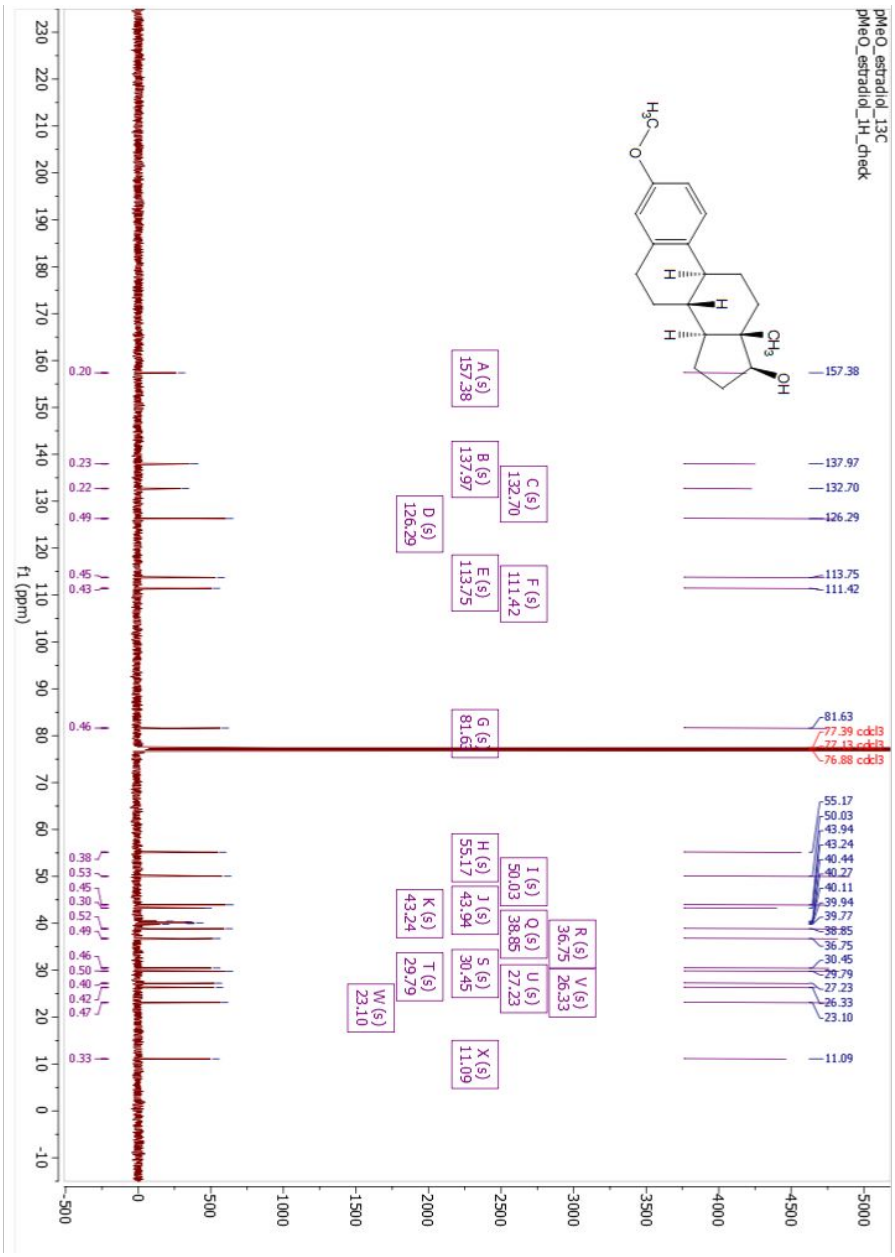


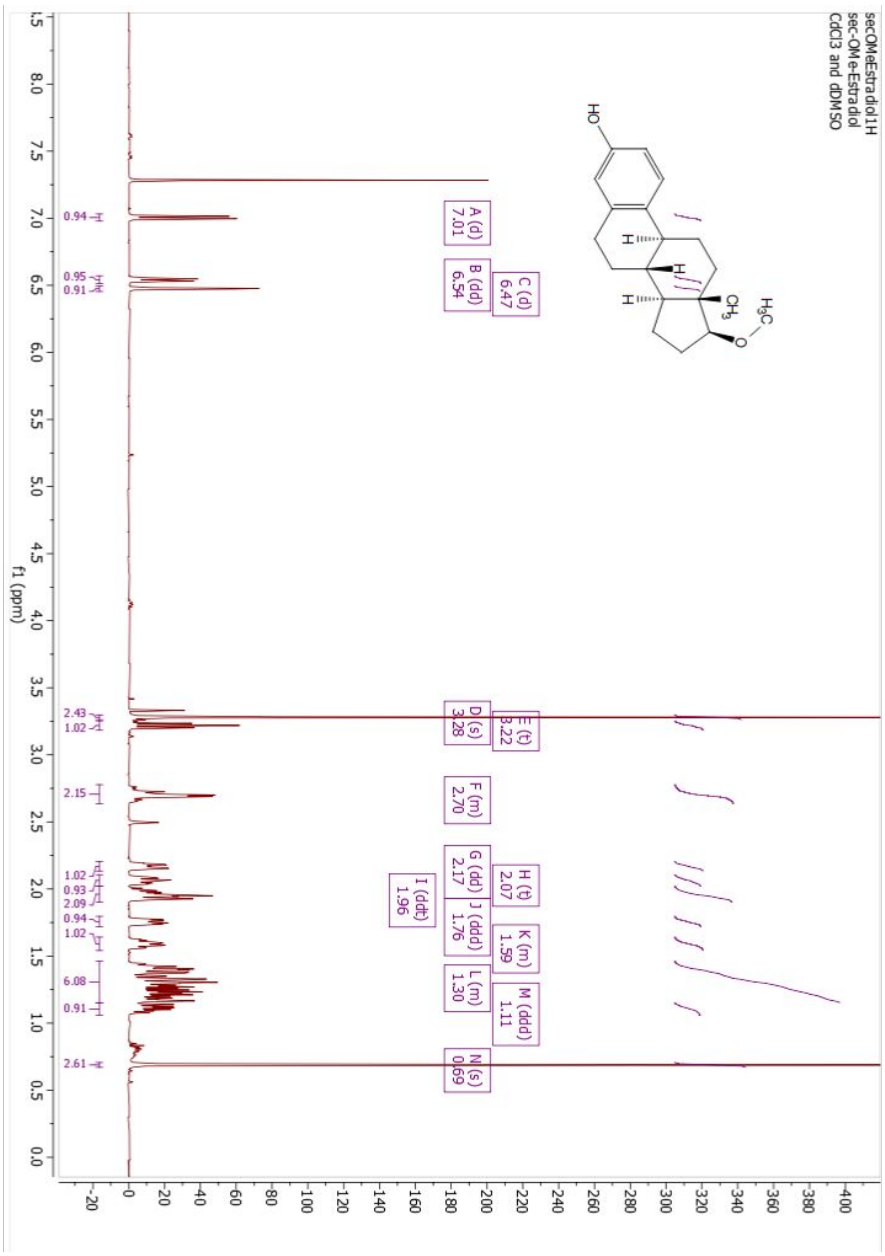
Figure S28: ^{13}C NMR of substrate 1d



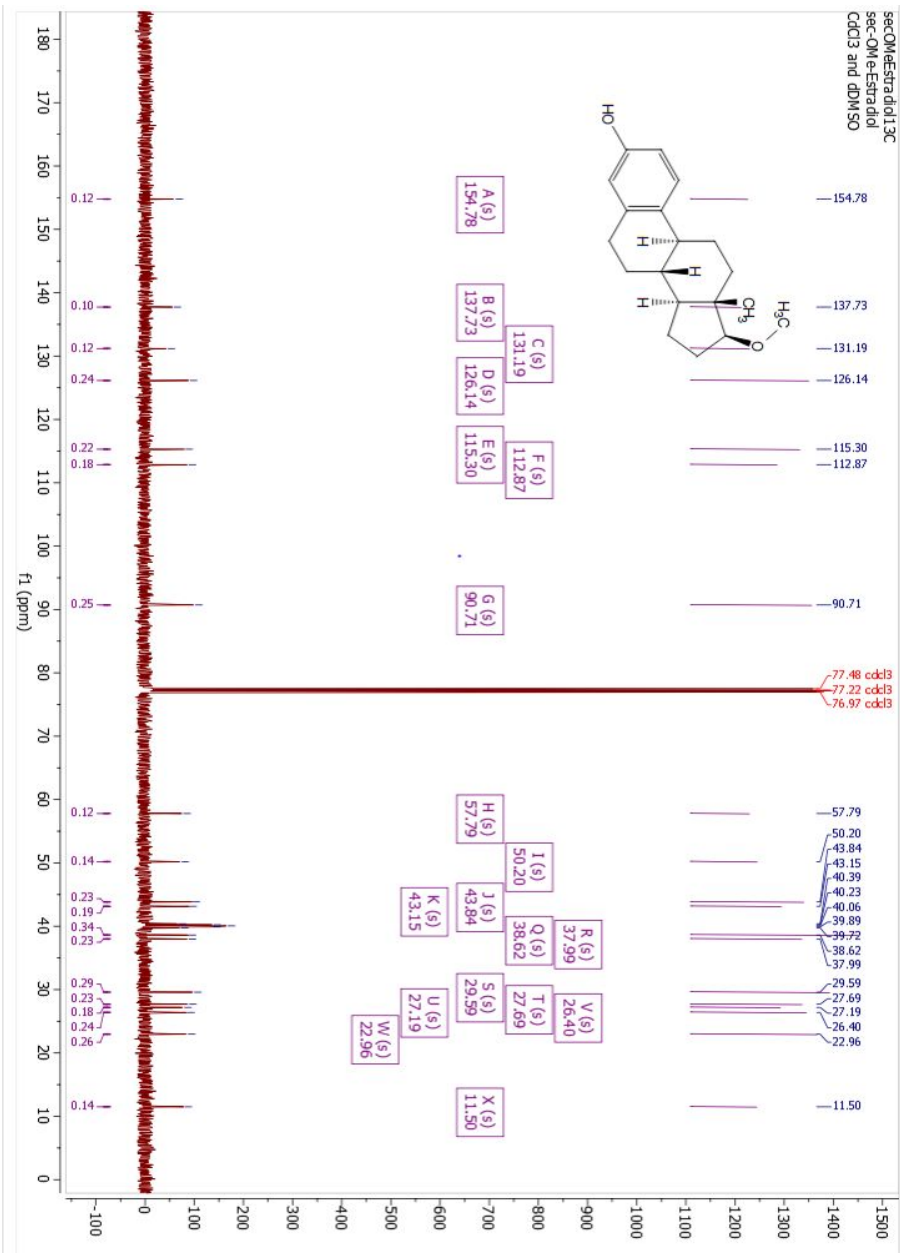
SI Figure S29: ^{1}H NMR of substrate 2a



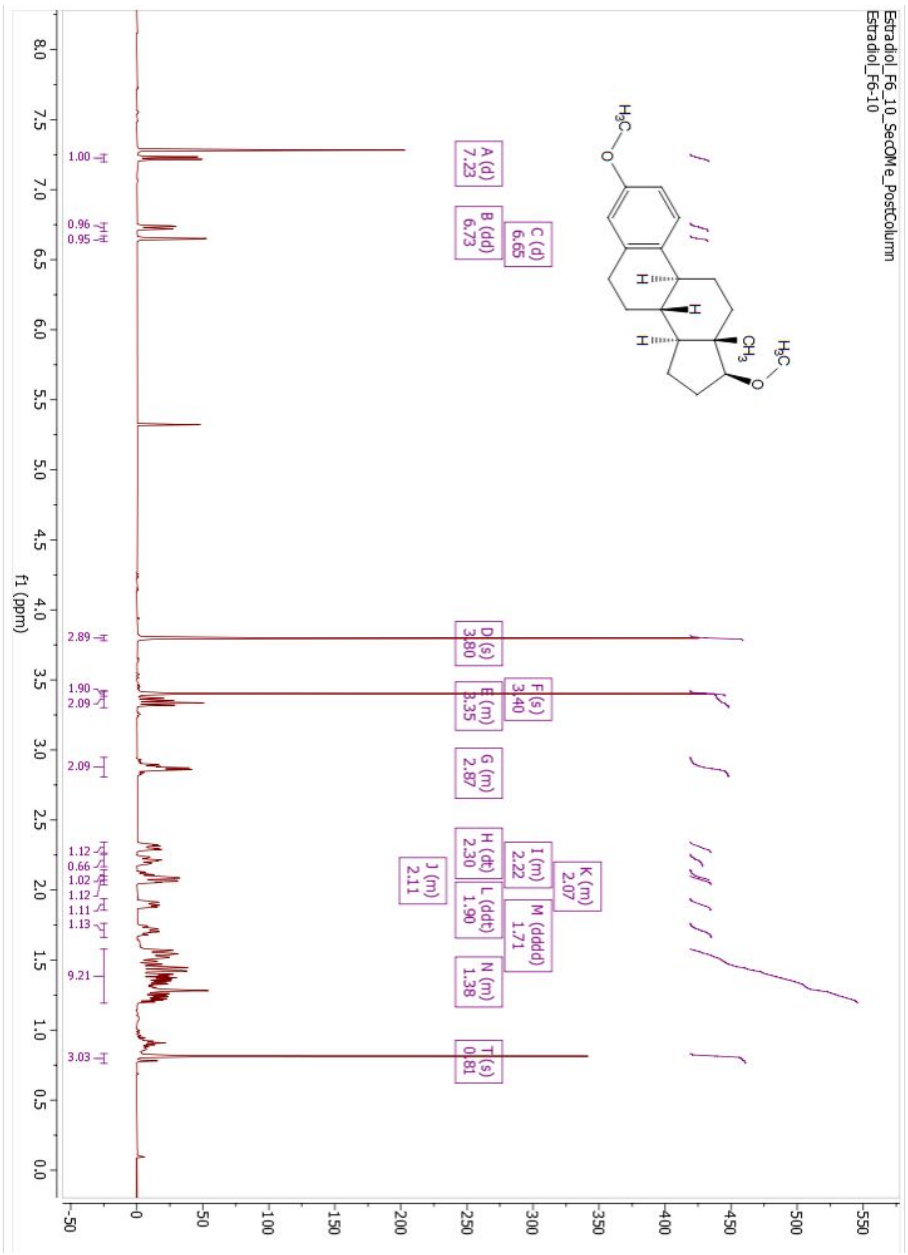
SI Figure S30: ^{13}C NMR of substrate 2a



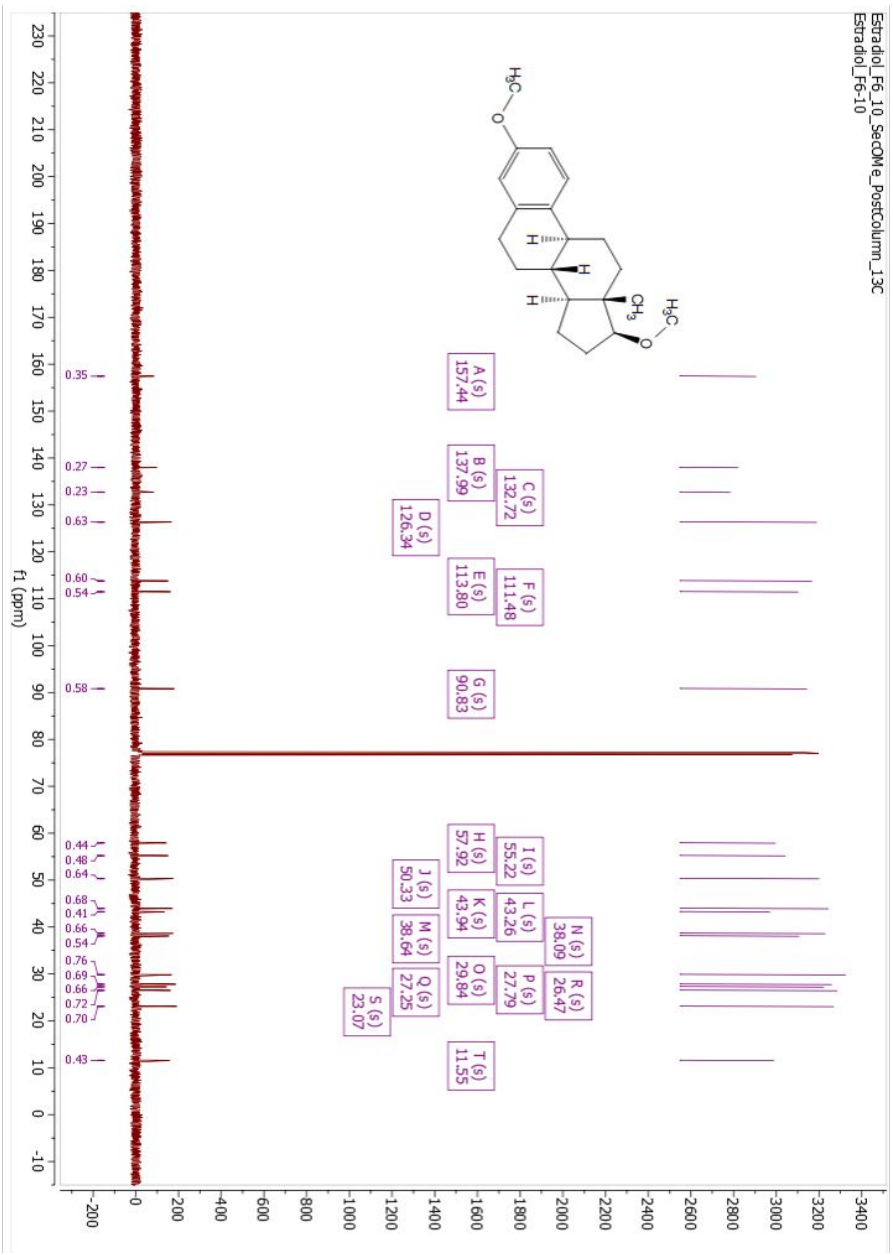
SI Figure S31: ^{1}H NMR of substrate 2b



SI Figure S32: ^{13}C NMR of substrate 2b



SI Figure S33: ^{1}H NMR of substrate 2c



SI FIgure S34: ^{13}C NMR of substrate 2c

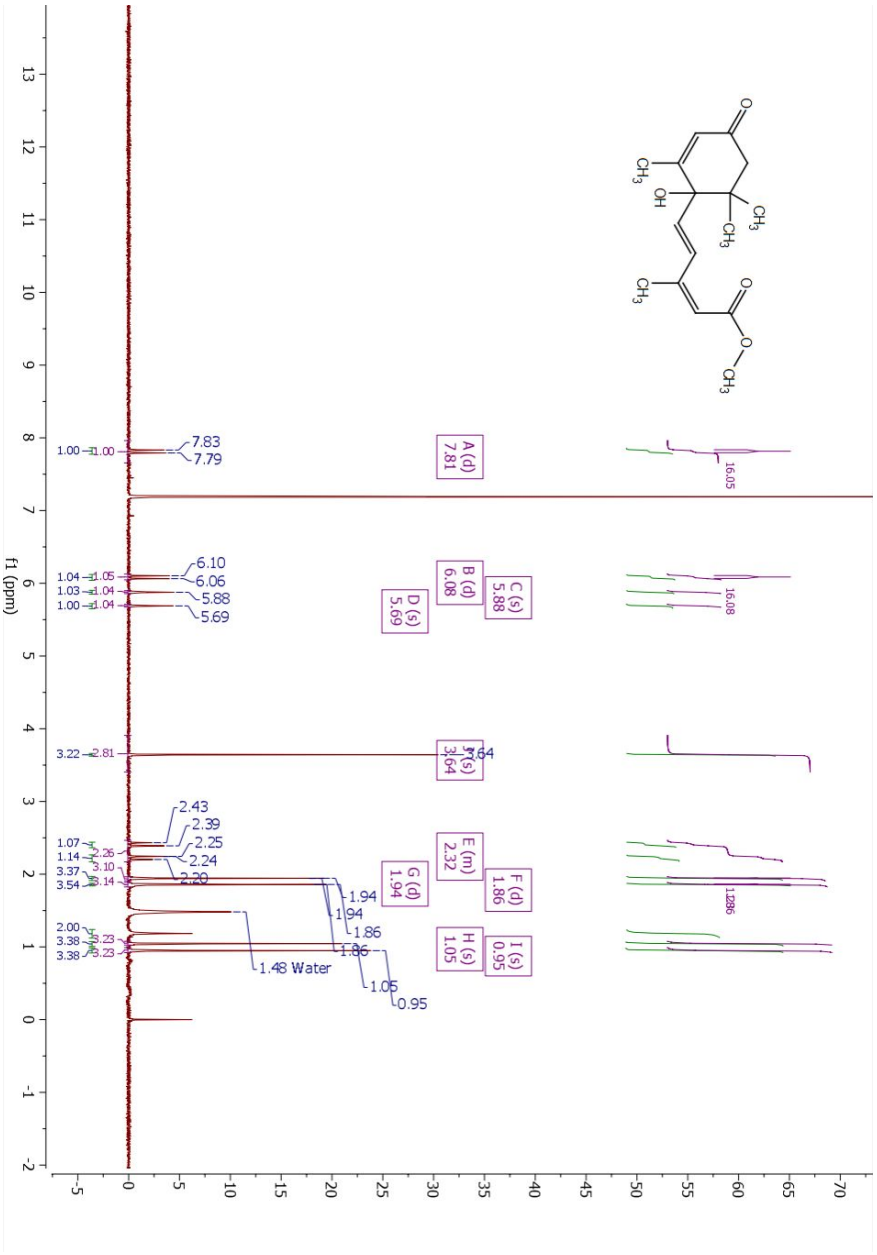


Figure S35: ^{1}H NMR of substrate 4a

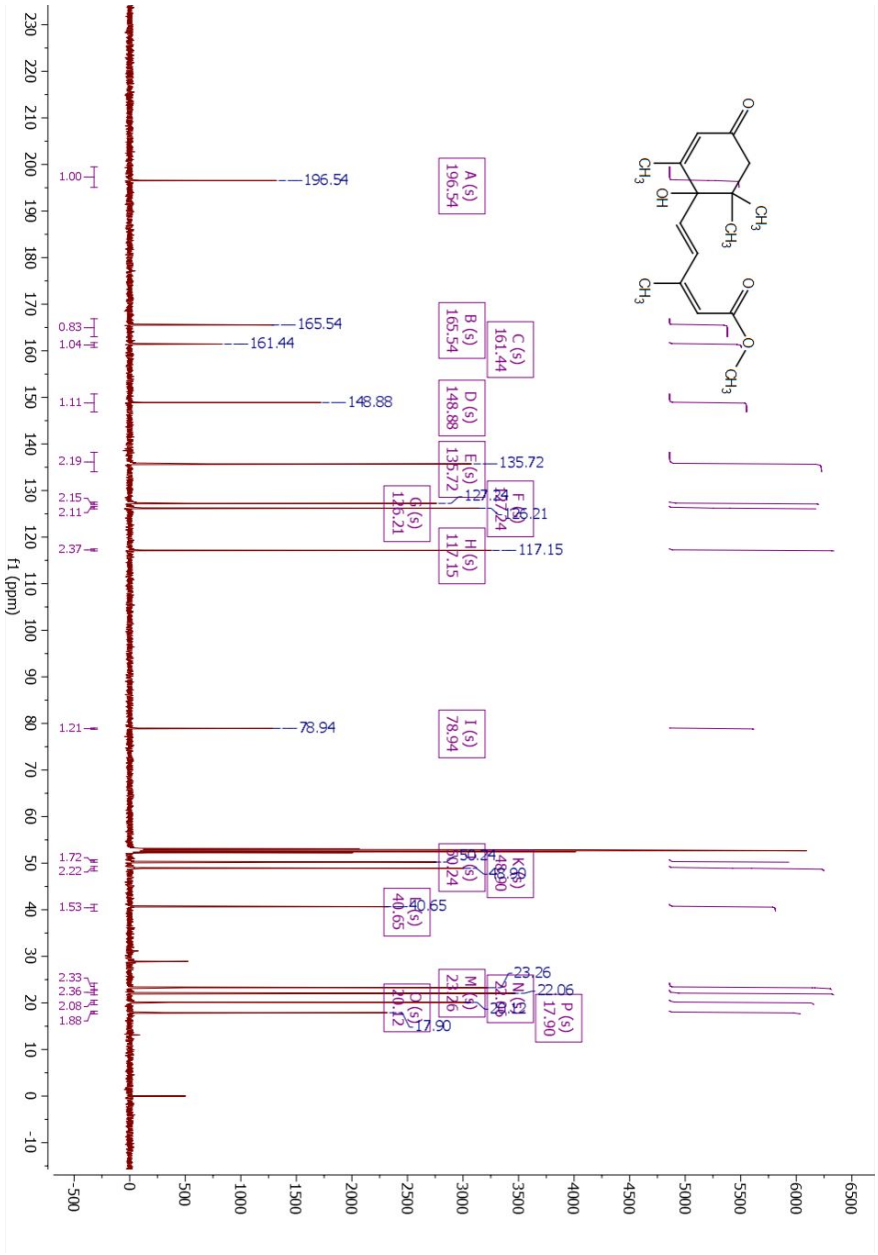


Figure S36: ^{13}C NMR of substrate 4a

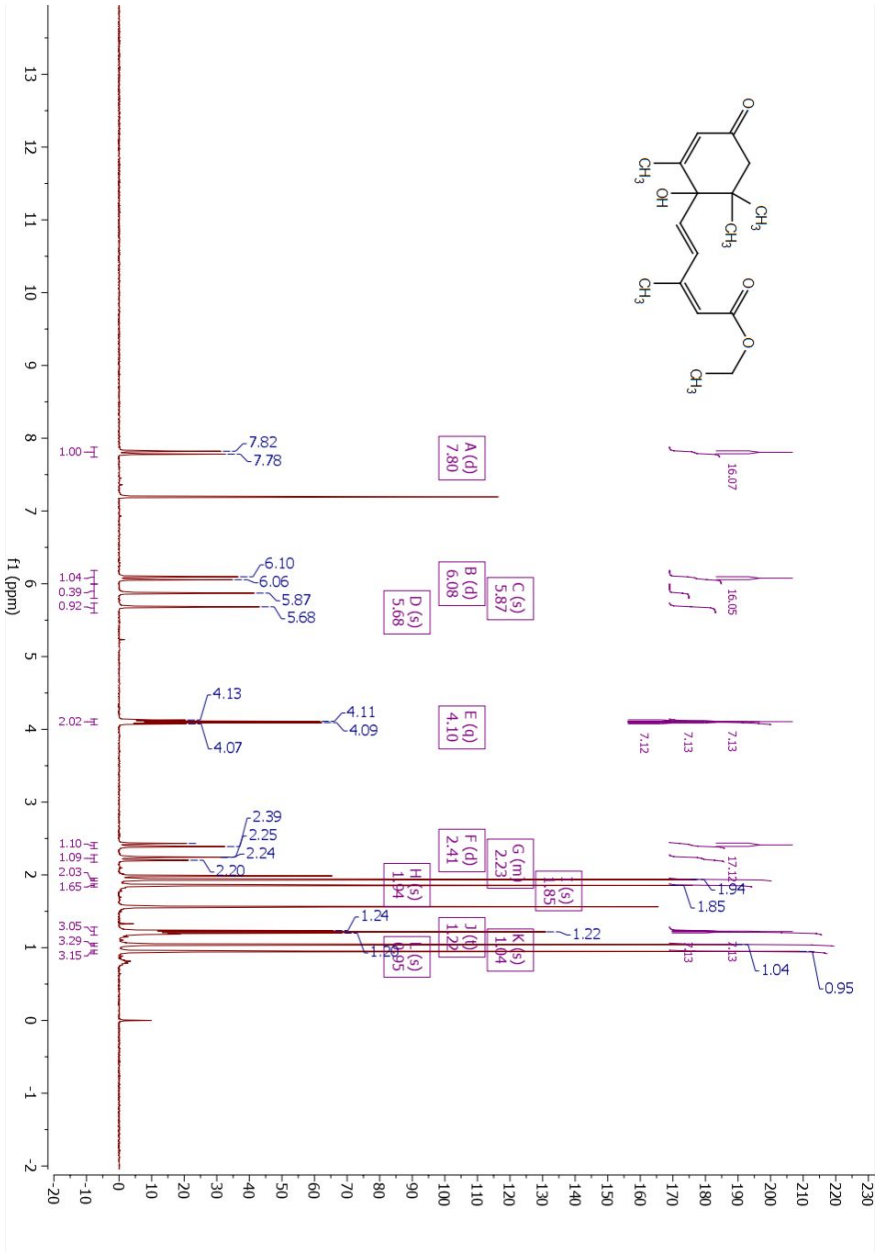


Figure S37: ^{1}H NMR of substrate 4b

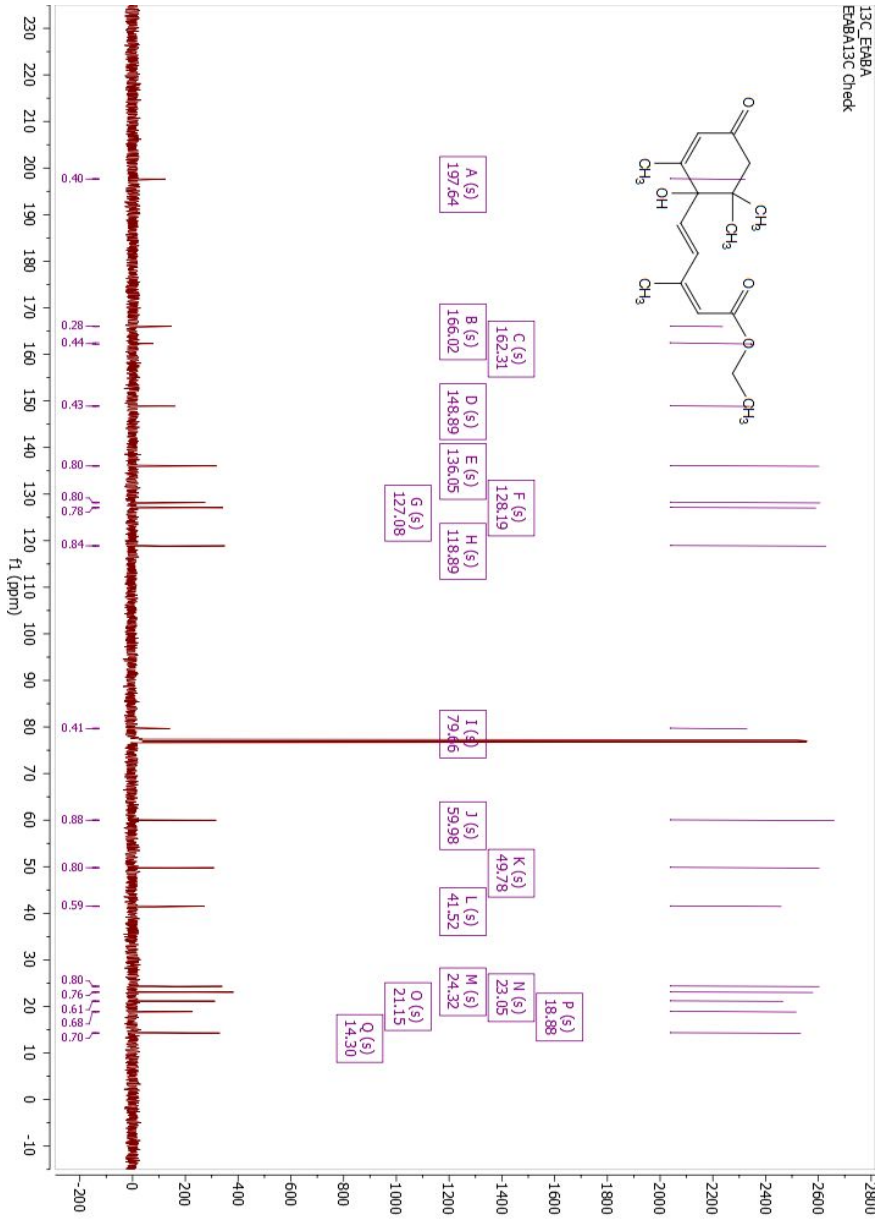


Figure S38: ^{13}C NMR of substrate 4b

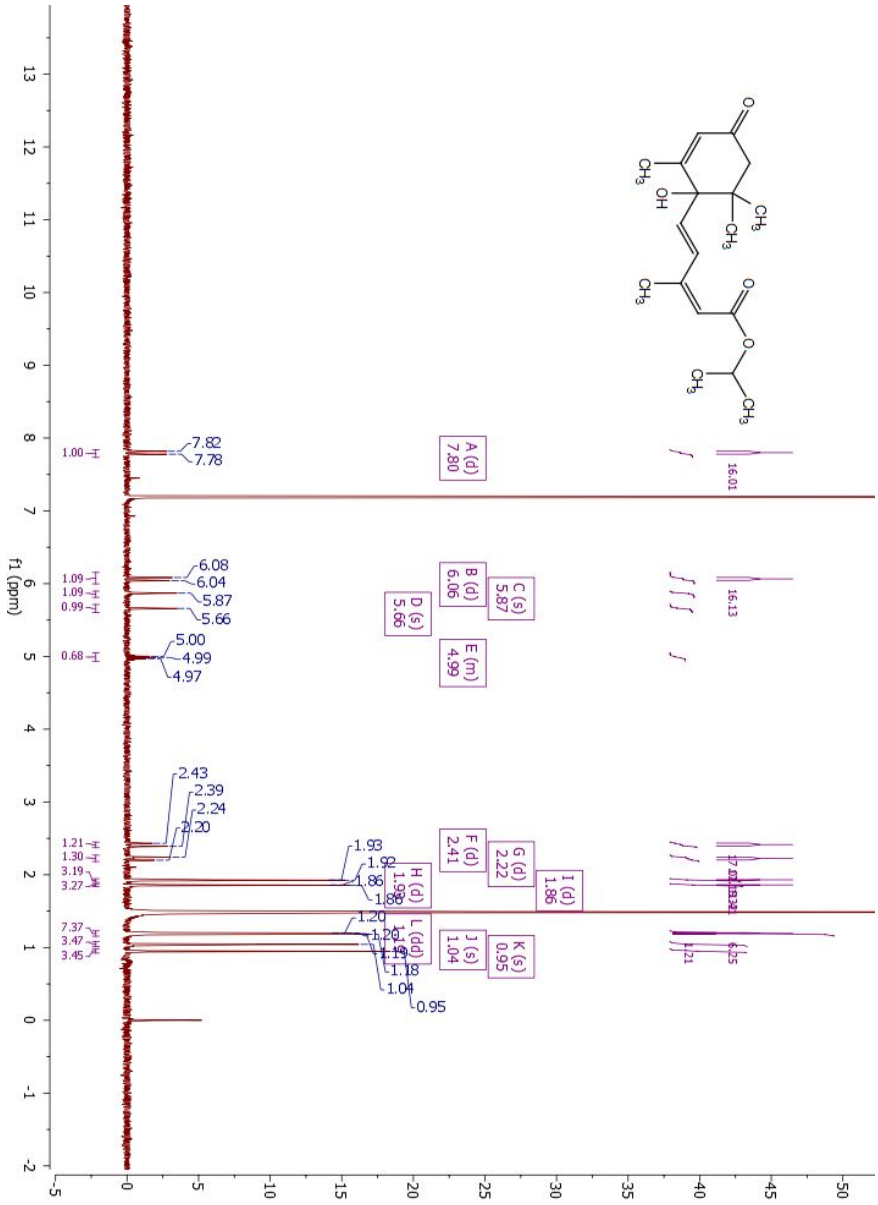


Figure S39: ^{1}H NMR of substrate 4c

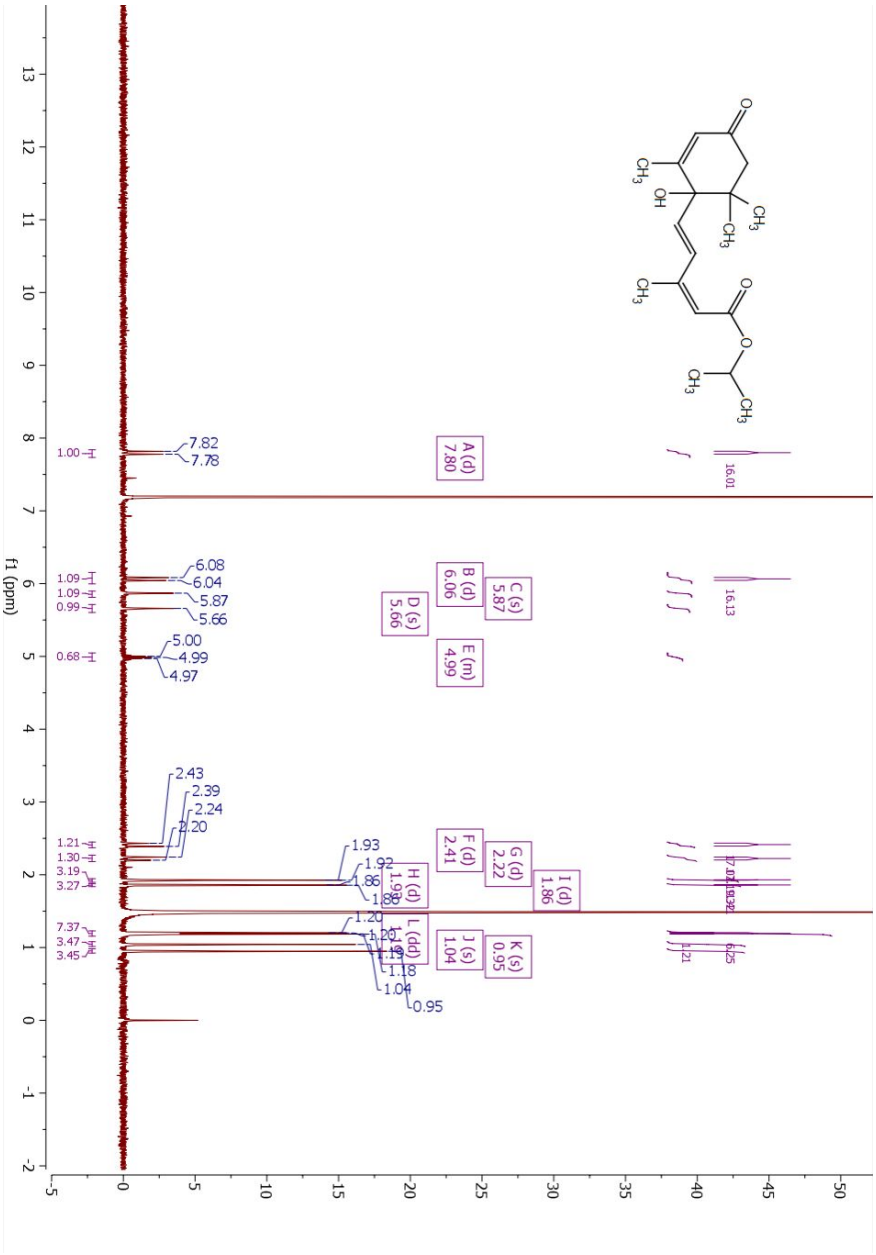


Figure S40: ^{13}C NMR of substrate 4c

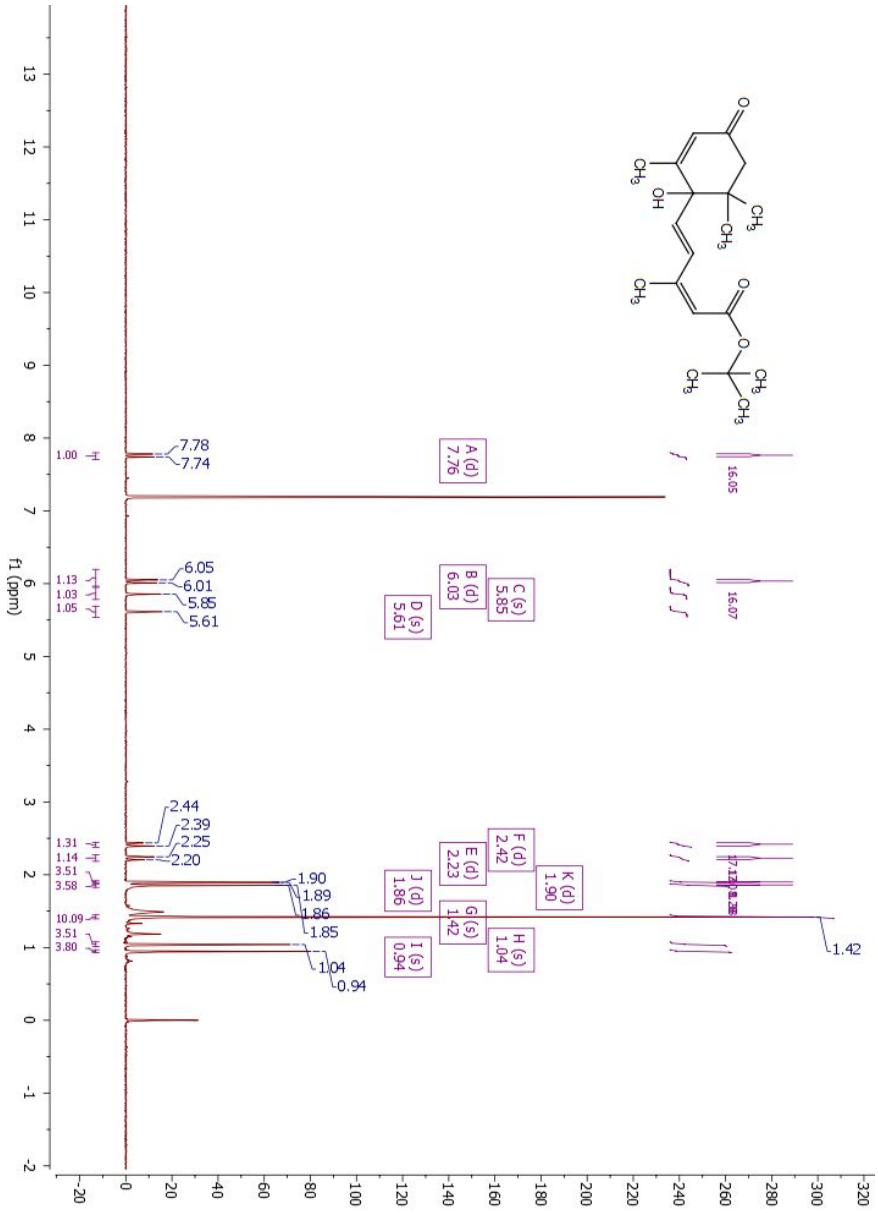


Figure S41: ^{1}H NMR of substrate 4d

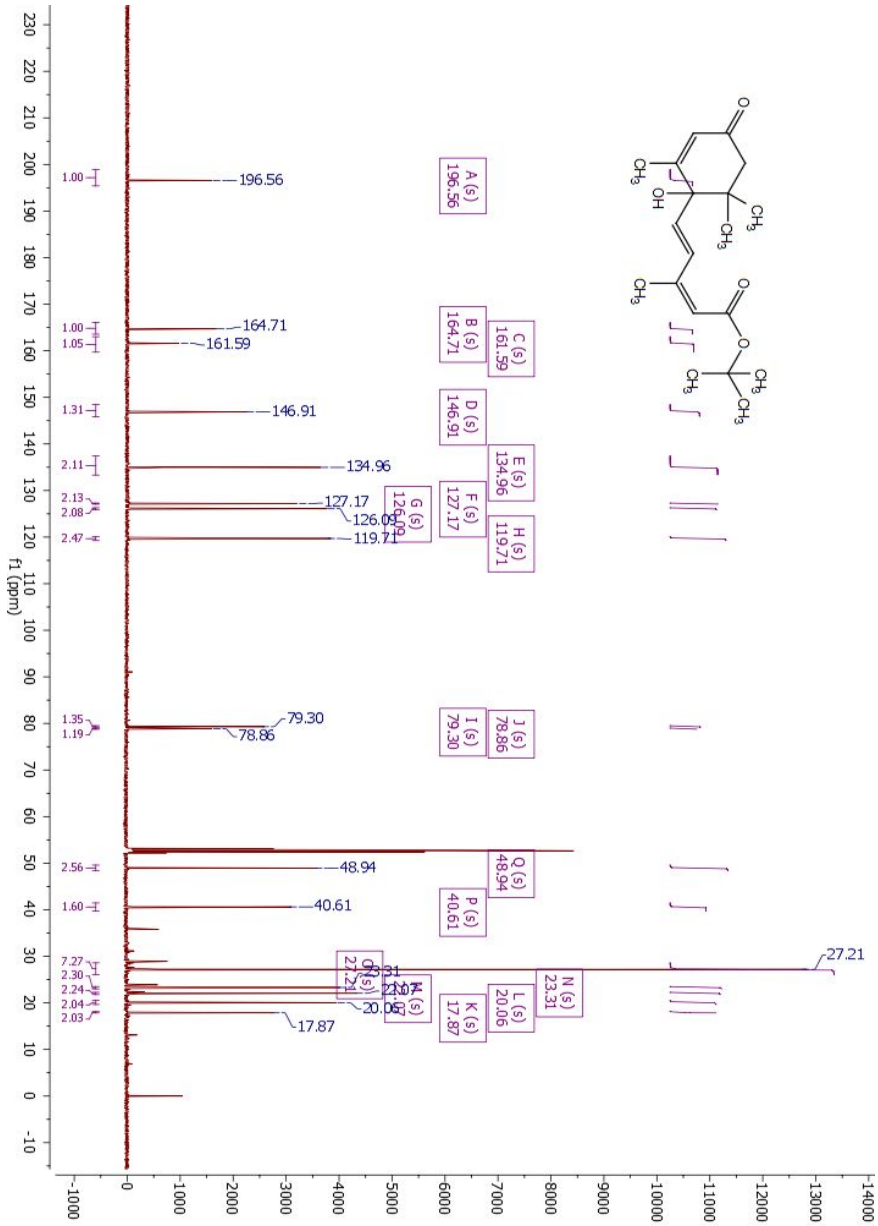
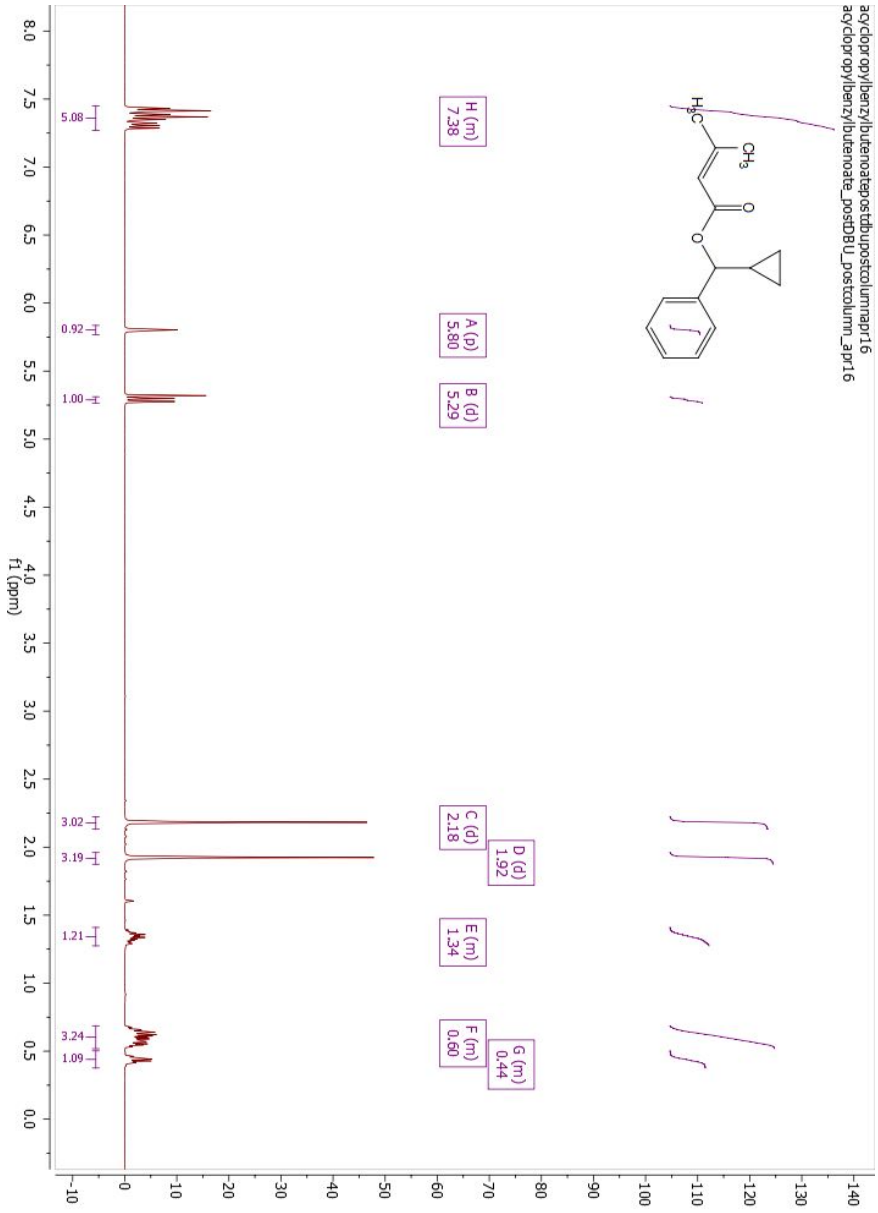
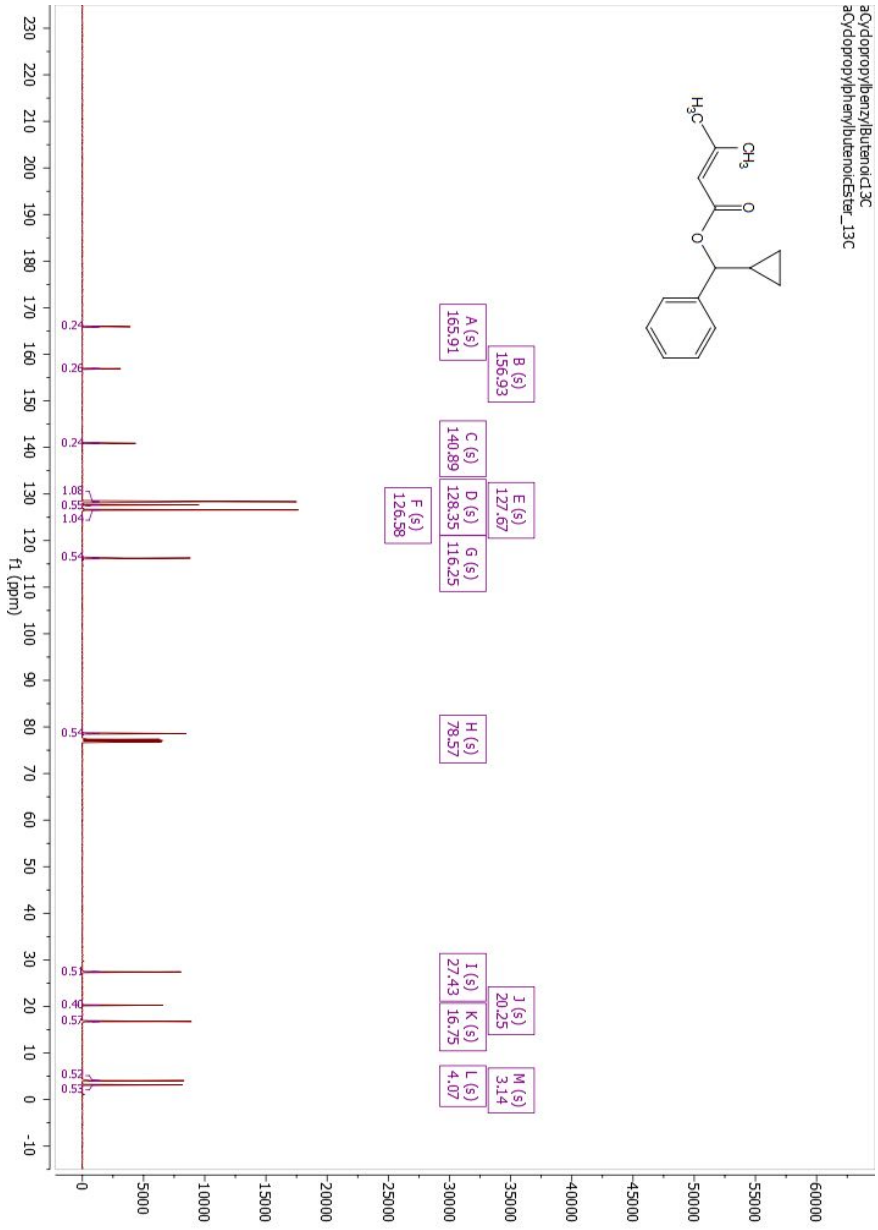


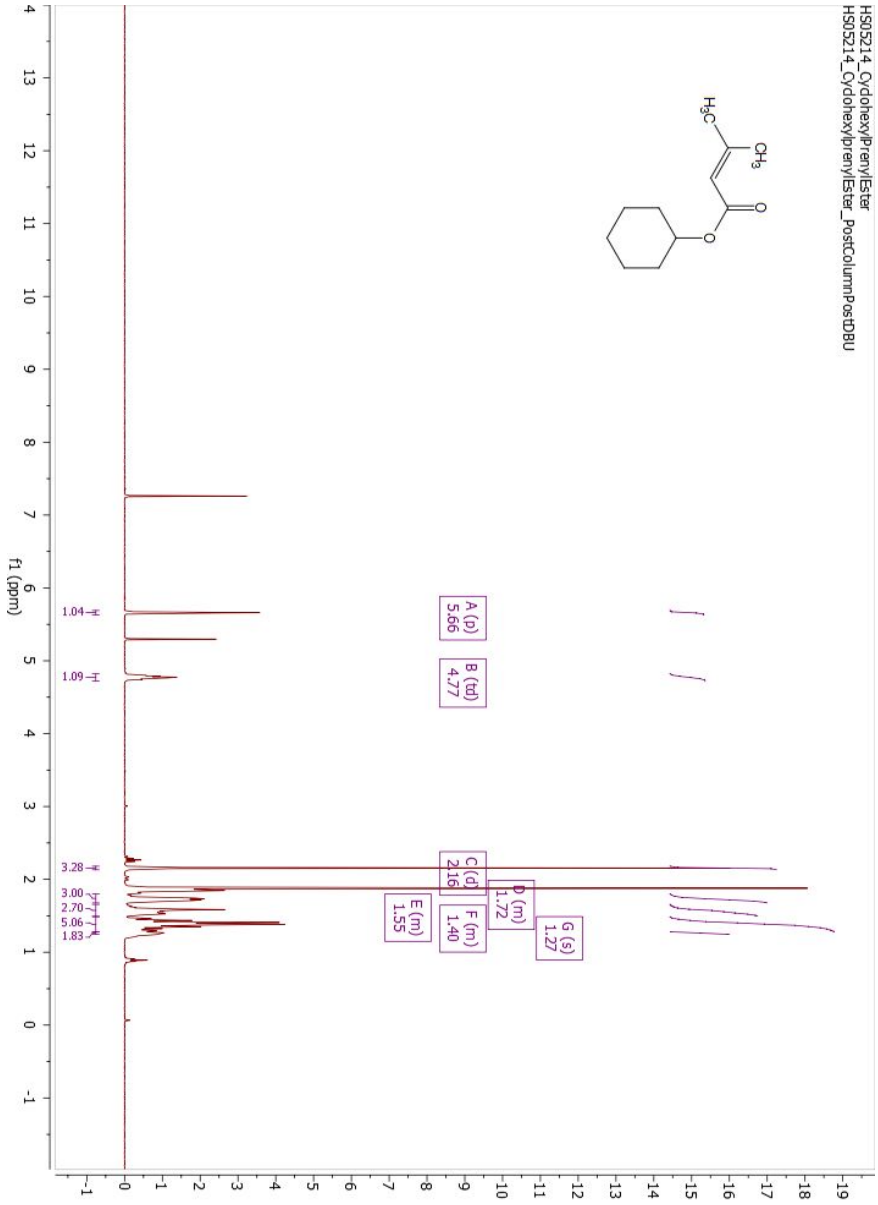
Figure S42: ^{13}C NMR of substrate 4d



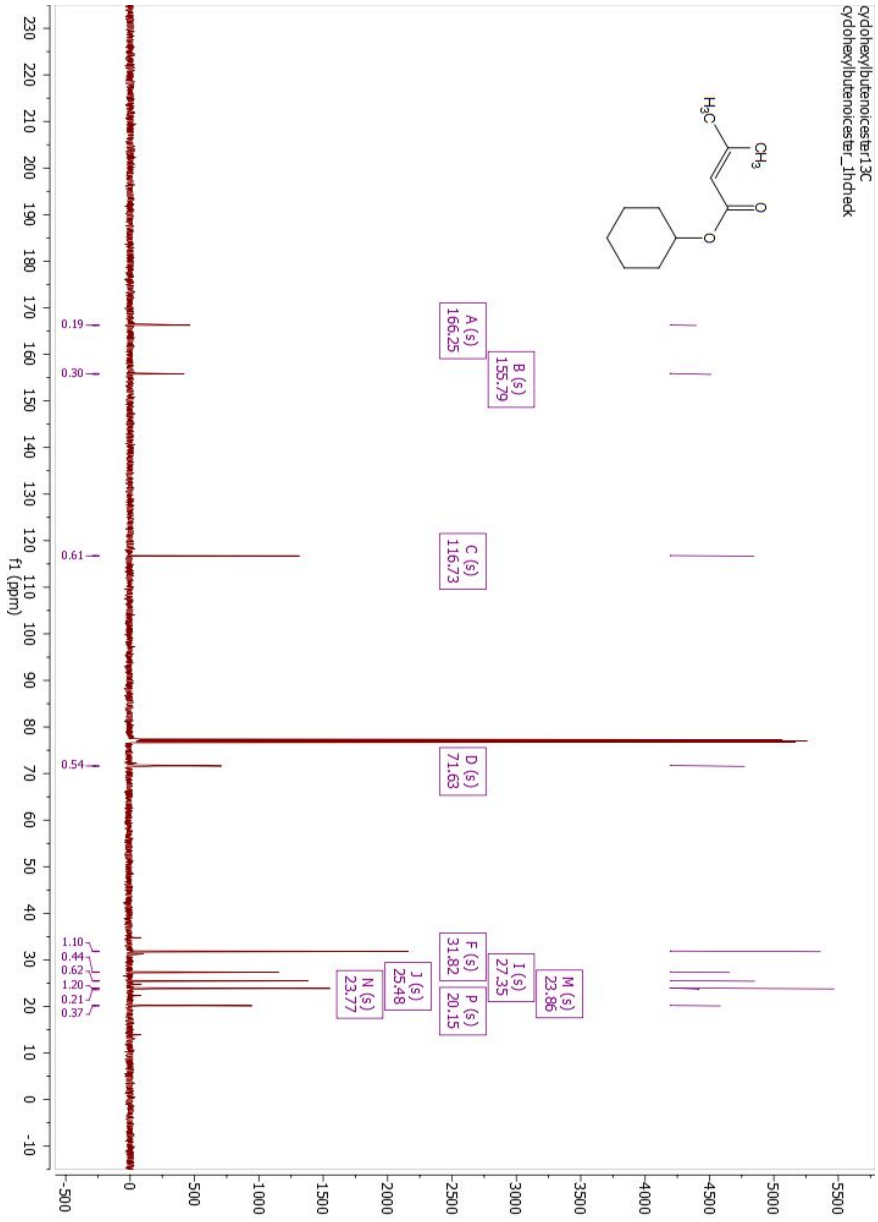
SI Figure S43: ^{1}H NMR of substrate 5



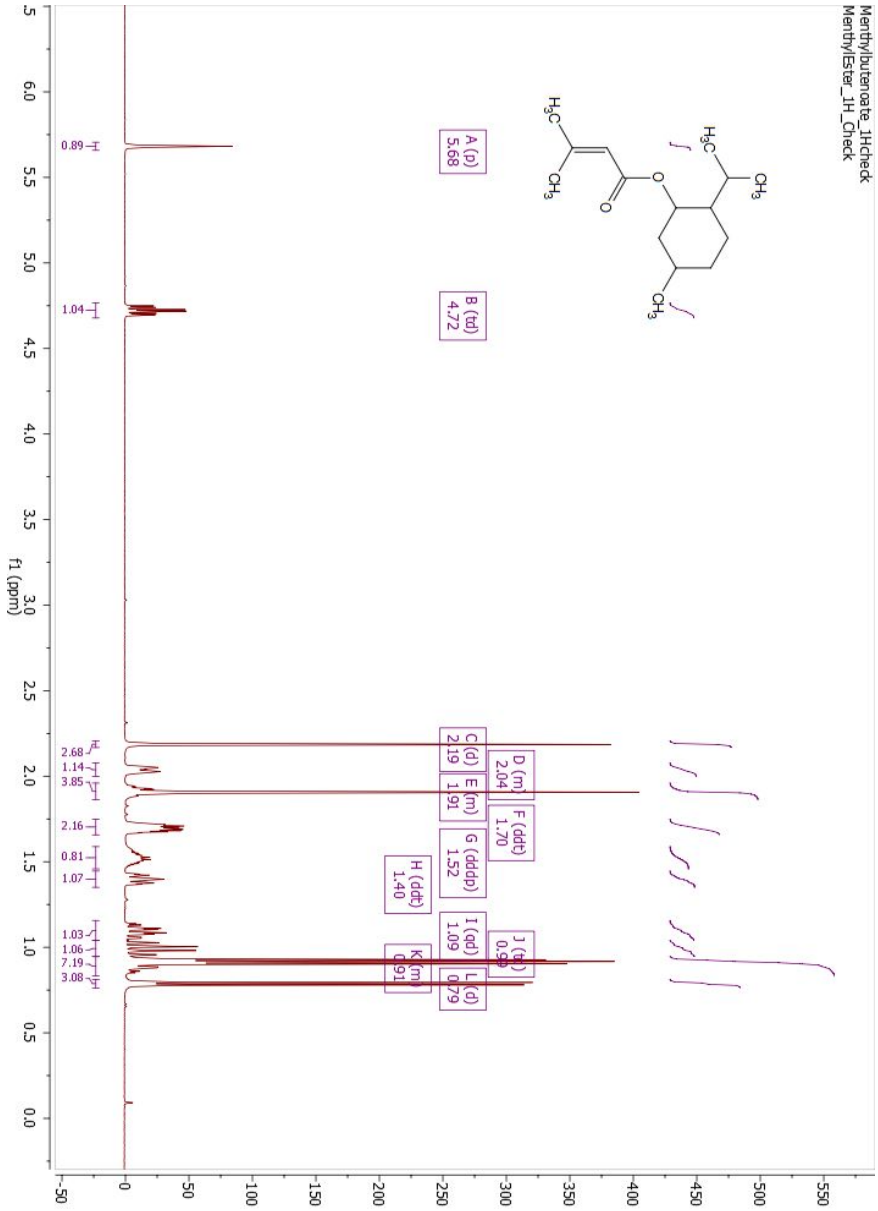
SI FIgure S44: ^{13}C NMR of substrate 5



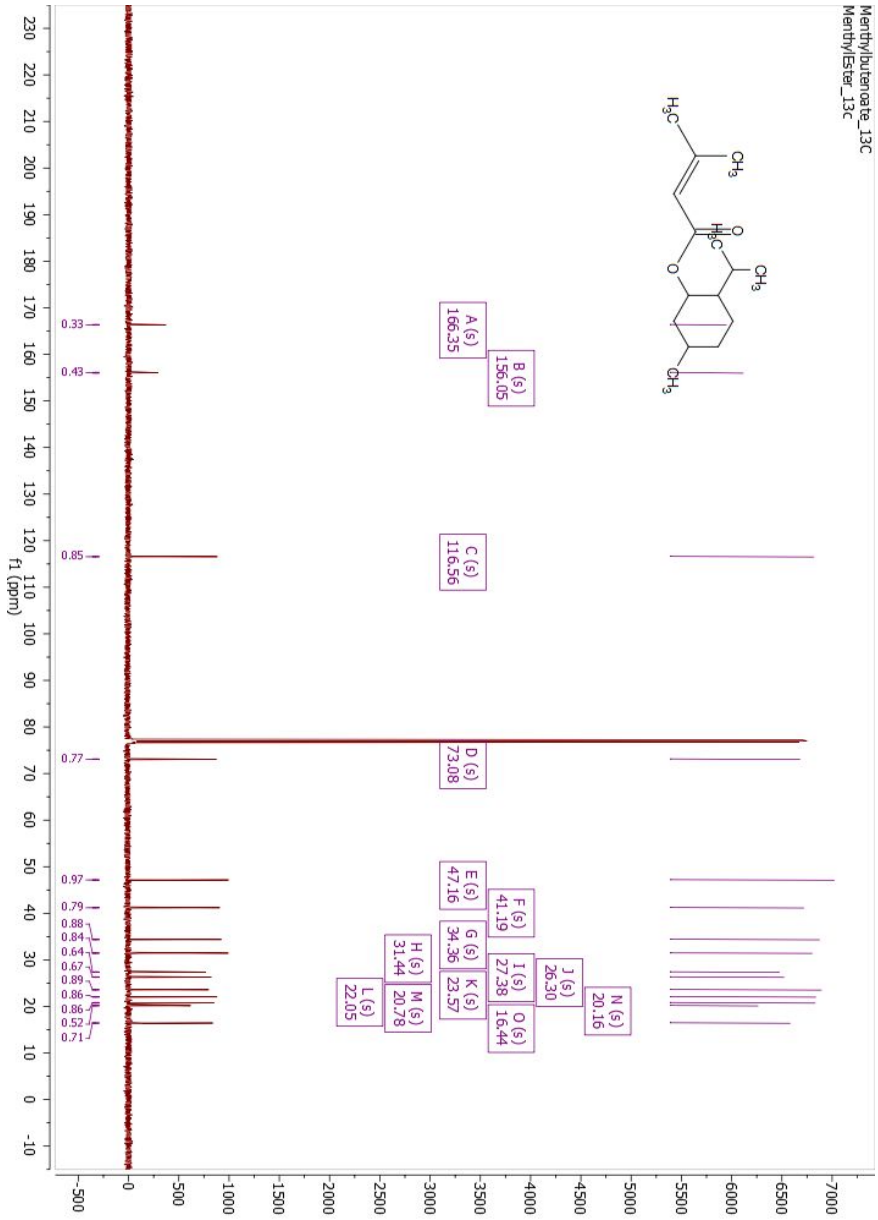
SI FIgure S45: ^{1}H NMR of substrate 7



SI FIgure S46: ^{13}C NMR of substrate 7



SI FIgure S47: ^{1}H NMR of substrate 8



SI Figure S48: ^{13}C NMR of substrate 8

VIII. References.

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