SUPPLEMENTARY INFORMATION

Differing Activities of Oxysterol-binding Protein (OSBP) Targeting Anti-Viral Compounds

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Cell Lines and Viruses

HEK293 STF (ATCC CRL-3249) and **HeLa** (ATCC CCL-2) were cultured in DMEM (Thermo 11995073) supplemented with 10% Hyclone (Fisher Sci SH3006603) and 1% penicillin-streptomycin (Thermo 15140122). **HCT-116** (ATCC CCL-247) was cultured in McCoy 5A media (Thermo 16600108) supplemented with 10% Hyclone and 1% penicillin streptomycin. **RD**, (rhabdomyosarcoma) cells (ATCC-CCL-136) were cultured in DMEM (Fisher Sci SH30081.0) with 10% FBS (Atlanta Biological S11550) and 1% penicillin-streptomycin (Gibco 15140-122). **Coxsackievirus A9** (strain CoxA9-01) and **Echovirus 2** (strain Echo2-01) were obtained from the Oklahoma State Department of Health Laboratory. They are clinical isolates, obtained from OK residents and typed by the OK State Department of Health and/or the Center for Disease Control and Prevention. All other identifiers have been stripped off. These viruses were passaged twice in RD cells, aliquoted in 1.0 mL amounts and stored in complete medium at -80 ºC. Each virus was titered on RD cells using a TCID-50 assay[1]. To allow M.O.I. to be determined, a conversion factor of 0.7 was used to change TCID-50 to pfu/mL.

General Cell Culture

All mammalian cell lines were cultured at 37 ºC in 5% CO_2. All handling of the mammalian cell culture was performed in a standard tissue culture hood using standard aseptic technique. Cell lines were cultured in the complete media described above. Cell culture stocks were aliquoted in 2 mL cryogenic vials (Corning 430659) in complete media with a DMSO concentration (5-11%) as specified by ATTC for each cell line and stored in liquid nitrogen vapor phase. Before beginning a new culture, the freezer stocks were thawed, diluted in 9 mL complete media and plated in Nunclon Delta 10 cm^2 dishes (VWR 10171744). After allowing ~16 hours for the revived cells to attach, the DMSO containing media was replaced with DMSO free complete media. All revived cultures were split at least twice prior to use in an experiment. Cell cultures were restarted approximately every 3-4 weeks. All cell based experiments reported used multiple restarted cell culture stocks in the independent experiments that make up the replicate results to ensure reproducibility between cell culture stocks. For experiments, cell cultures were used with a confluency of ~70%. The cell cultures were not allowed to become superconfluent, and the cellular morphology and proliferation rate of the cell culture was carefully tracked to identify any abnormalities; any cell culture showing the slightest abnormalities were discarded and the cell line restarted from frozen stocks. For experiments, cells were allowed to recover from seeding a minimum of 16 hr prior to the start of an experiment. The adherent mammalian cell lines are split every ~2-3 days with the following general procedure: the complete media is removed via aspiration and the cells are gently washed with 5 mL of 1X PBS. TrypLE™ Express (Gibco 12605-010) trypsin reagent (2.5 mL for 10 cm^2 plate) is added and incubated for approximately 10 min at 37 ºC. After ~10 mins, 7.5 mL of the complete culture media is added to inactivate the TrypLE™ Express reagent. Cells were counted using a TC20™ Automated Cell Counter (BioRad), by combining 10 µL of cell solution with 10 µL Trypan Blue stain (Thermo 15250061).

Cell Lysis (AC Freeze/Thaw Lysis)

Adherent cells were cultured in Nunclon Delta 10 cm^2 dishes (VWR 10171744) and lysed by removing the media, washing with 1X PBS, followed by addition of 1 mL PBS and scraping. Cells were collected in a 1.5 mL Eppendorf brand centrifuge tubes (Cat. No. 022363204) and spun down

at 14,000 x g for 45 seconds. Supernatant was removed, and the cells were resuspended in 50 µL of AC Lysis buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 5% glycerol, 0.8% NP40, 1mM DTT, 50 mM HEPES, 25 mM NaF, 1 mM Na_3PO_4) with 3X HALT/EDTA protease inhibitor (Thermo 78438) and 0.2 mM phenylmethylsulfonylfluoride (Goldbio). The cells were then frozen in liquid nitrogen and thawed in a 37 °C bead bath three times with gentle vortexing between thaws, followed by a 14,000 x g spin for 15 minutes. Supernatant was transferred to a new tube and a portion was taken for protein quantification using a Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate #5000006, BSA-Santa Cruz sc-2323). After protein quantification, the lysates were diluted to the desired concentration using AC lysis buffer and 4X Laemmli buffer (1 M Tris pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, and 0.2% bromophenol blue), followed by dry bath heating at 95 °C for 10 minutes. Adherent cells cultured on 6-well plates (Greiner 657160) were lysed by removing media, washed with 1X PBS, followed by adding 0.5 mL TrypLE™ Express (Gibco 12605-010) and incubated at 37 °C for 5 minutes. TrypLE™ Express was neutralized using 0.5 mL of media and cells were then transferred to a 1.5 mL Eppendorf tube and spun down at 14,000 x g for 45 seconds. Supernatant was removed, and 1 mL of PBS was added to wash the cells. Cells were spun down at 14,000 x g for 45 seconds, supernatant was removed, and the cells were resuspended in 50 µL of AC lysis buffer. Freeze/thaw method was continued as described above.

OSBP Expression in Cells Upon Compound Treatments

HCT-116 or HEK293 cells were seeded out $5x10^5$ cells per well into a 6-well plate and incubated at 37 ° C for 20 h. Cells were then treated with either DMSO (Sigma 472301), OSW-1 (1 nM), Taxol (10 nM), Itraconazole (10µM), TTP (10µM), or T-00127-HEV2 (10µM) for 24 h (**Fig. 1A**). Compound stock solutions were diluted in complete media before treating the cells. For coincubation experiments (**Fig. 4C**), cells were seeded as described above and treated with OSW-1 (1 nM) co-incubated with either Itraconazole (10µM), TTP (10µM), or T-00127-HEV2 (10µM) for 24 h. After compound treatment, cells were lysed according to the lysis procedure in the Supporting Information.

Western Blotting

SDS-PAGE gels (8.5%) containing 25 µg of protein per well were transferred to 0.45 µm nitrocellulose (Bio-Rad 1620115) using constant voltage (100V) for 1 h at 4 °C in 1X transfer buffer with 10% ethanol. After transferring, the nitrocellulose membrane was blocked with 5% milk in 1X TBST at room temperature for 30 minutes. The membranes were then washed 3 times, 5 minutes each, with 1X TBST. Primary incubation with antibodies was done overnight at 4 °C. After primary incubation, the blots were washed 5 times, 5 minutes each, with 1X TBST and then incubated in secondary antibody in 1% milk TBST for thirty minutes at room temperature. After secondary antibody incubation, the blots were washed 5 times, 5 minutes each, with 1X TBST and then once with 1X TBS for 10 minutes. TBS was removed, and the blots were incubated in Clarity™ Western ECL substrate (Bio-Rad 1705061) and imaged on the Bio-Rad ChemiDoc™ Touch Imaging System using the chemiluminescence setting with 2x2 binning. Ladder images were taken using the colorimetric setting. After development, the membranes were washed with 1X TBST two times for 5 minutes each. 1:1000 β-actin HRP (Santa Cruz sc-47778 HRP) in 1% milk TBST was added as a loading control and incubated for 1.5 hr at room temperature. Blot development was then performed as described above. The primary antibody used for Western

blotting was 1:500 OSBP A-5 (Santa Cruz sc-365771) and the secondary antibody used was 1:3000 goat anti-mouse IgG1-HRP (Santa Cruz sc-2060).

Cloning and Plasmids

The cloning and plasmids generated for the OSBP and ORP4 transfection for the [3H]-25-OHC binding assay were performed as previously described by Roberts et al., 2019 in Supplemental Information[2].

([^{3}H]-25-OHC) Charcoal/Dextran Binding Assay

The [^{3}H]-25-OHC binding assay was run according to the protocol outlined by Burgett et al[3].

Cytotoxic Assay Protocol

HCT-116 and HEK293 cells were seeded at 2,000 cells per well and HeLa cells were seeded at 5,000 cells per well into opaque 96-well Falcon plates (VWR 25382-208). Cells were allowed to rest for 20 hr before treatments. Day zero control plate was created by adding 25 µL of media containing either 0.1% DMSO or 1% DMSO and 20 µL of cell titer blue (Promega G8081) to each of the wells containing cells. Incubated at 37 °C, 5% CO_2 for 1 h and 30 mins. Plates were read using a GloMax® Discover Microplate Reader using the Cell Titer Blue protocol. Remaining plates with cells were treated with various dilutions of compounds and incubated at 37 °C for 48 hours under 5% CO_2. After the incubation time, 20 µL of cell titer blue was added to the wells and the plates were incubated and read as incubated above. Control plate was subtracted from the treatment plate and the values were analyzed using GraphPad Prism software. Co-incubation experiments were done using the same protocol described above with the following changes. 10µM of ITZ, TTP, or THEV was added to all the wells during the 48 hr treatment and the OSW-1 concentration was varied as indicated on the representative cytotoxic assay curves.

Trypan Blue Cell Viability Experiments

HCT-116 cells were seeded out $5x10^5$ into 6-well plates and left to rest for 20 hrs. The cells were treated with DMSO (Sigma 472301), 1 nM OSW-1, 10µM Itraconazole, 10µM TTP, 10µM THEV, or a combination of treatments for 24 hrs. Cells were washed with 1X PBS and then incubated in 0.5 mL TrypLE™ Express for 5 mins at 37 °C. TrypLE™ Express was neutralized using 0.5 mL of fresh media and cells were counted on a TC20™ Automated Cell Counter (BioRad) by combining 10 µL of cell solution with 10 µL Trypan Blue stain (Thermo 15250061). The remaining cell solution was lysed and analyzed by Western blot.

Immunofluorescence

HCT-116 cells were seeded at 50,000 cells onto sterile 18 mm cover slips in 12-well plates for treatments lasting 24 hrs. The cells rested for 24 h before treatment to ensure attachment. Once treatments were completed, media was removed and cells were washed with warm 1X PBS. PBS was removed and 0.5 mL of freshly prepared 4% paraformaldehyde in PBS was added. Cover slips were incubated at 37 °C for 20 minutes and then the paraformaldehyde was removed followed by three 1X PBS washes. Permeabilization of the cells was done with 0.5 mL of 0.5% Triton X-100 in PBS at room temperature for 10 mins. 1X PBS was used to wash the cells three times. Image-iT FX signal enhancer (Thermo I36933) was added onto the cover slips, and incubated at room temperature for 30 mins followed by three 1X PBS washes. Coverslips were blocked with 0.5 mL of 1% BSA in PBS at room temperature for 30 mins followed by 3 washes with 1X PBS. Primary

antibody was added and the slips were incubated overnight at 4 °C. The primary antibody solution was removed and the cover slips were washed three times with 1X PBS. The secondary antibody was incubated in darkness at room temperature for one hour. The secondary antibody solution was removed and the slips were washed 3 times with 1% BSA-PBS, 3 times with 1X PBS, and then soaked the cover slip in 300 nM DAPI (Thermo D1306) solution for 10 minutes. The slips were mounted onto glass slides using VECTASHEILD HardSet Antifade mounting media (VECTOR labs H-1400). Slides were stored at -20°C until imaging was conducted. Primary antibodies used were 1:100 OSBP1 1F2 (Novus NBP2-00935) and 1:500 TGN46 (Novus NBP1-49643). Secondary antibodies used were 1:500 goat anti-mouse IgG H&L Alexa Fluor® 488 (Abcam ab150113) and donkey anti-rabbit IgG H&L Alexa Fluor® 594 (Abcam ab150076). Imaging was done with a Lecia SP8 using a 63x objective with 2x digital zoom. Images were analyzed with ImageJ software.

Antiviral Experiments

HeLa cells were grown to <75% confluency (healthy log phase cells) in complete media, DMEM (Hyclone SH30081.0) with 10% FBS (Atlanta Biological S11550) and 1% penicillin-streptomycin (Gibco 15140-122). For experiments, cells were trypsinized, counted using a hemocytometer, and seeded into 20 wells of two 24-well trays (Falcon 3047) with 1.0 x 10^5 cells per well, in 1.0 mL complete media. Each treatment is performed using quadruplicate wells (n=4) and each virus was on a separate plate. After seeding, cells were incubated 20 hr at 37 ºC, 5% CO_2, at which point cells have grown to a near confluent monolayer.

For the antiviral continual treatment experiments, (**Fig. 2A**), the media was gently removed from each well and CoxA9-01 or Echo2-01 viruses, diluted in serum-free DMEM with a M.O.I. of 1.0 was added to the culture. The 1.0 x 10^5 cells per well was assumed to double during incubation so 2.0 x 10^5 pfu/well of virus was used for an M.O.I. of 1.0. The virus and cells were incubated for 30 minutes at 37 ºC, 5% CO_2. Then, the virus inoculum was removed, and the culture washed one time with 1.0 mL of serum-free media per well. 1 mL of media was added to each well containing either 10,000 nM ITZ, TTP, THEV, or 10 nM OSW-1. The infected cells were then incubated in media with the indicated compound for 10 h at 37 ºC, 5% CO_2. After 10 h, the plate was stored at -80 ºC until the TCID-50 titration. This experiment was performed independently three times to generate the data in the figure.

For the antiviral washout treatment experiments, (**Fig. 2B**), cells were seeded as described above. After 20 hr incubation the media was gently removed from each well, and 1 mL of media was added containing either 10,000 nM ITZ, TTP, THEV, or 10 nM OSW-1. Cells were incubated for 6 hr, after which time the media was removed and cells were gently washed three times with 1.0 mL of FBS-free DMEM media. Media was replaced with complete compound-free media and cells were allowed to incubate and recover from compound treatment for 24 hrs. After the media was removed, CoxA9-01 or Echo2-01 viruses, diluted in serum-free DMEM with a M.O.I. of 1.0 was added to the culture. The 1.0 x 10^5 cells per well was assumed to double and double again during incubation so 4.0 x 10^5 pfu/well of virus was used for an M.O.I. of 1.0. The virus and cells were incubated for 30 mins at 37 ºC, 5% CO_2. Then, the virus inoculum was removed, and the culture washed one time with 1.0 mL of serum-free media per well. Then, 1.0 mL of complete media was added to the well, and the infected cells were then incubated for 10 hr at 37 ºC, 5% CO_2.

After 10 h incubation, the plate was stored at -80 ºC until processing. Then, the plates were rapidly thawed, the cells in media were scraped from the wells into sterile 1.5 mL centrifuge tubes

and the suspension then centrifuged at 10,000 x g at 4 ºC to produce the virus containing supernatant, which is assayed for TCID-50 titration on sub-confluent RD cells. This experiment was performed independently three times to generate the data in the figure. The TCID-50 titration was performed according to the protocol described by Reed et al.[1] This experiment was performed independently three times to generate the data in the figure.

General Synthetic Methods

All reactions were performed in oven-dried glassware under a positive pressure of nitrogen unless noted otherwise. Flash column chromatography was performed as described by Still et al.[4] employing E. Merck silica gel 60 (230-400 mesh ASTM). Thin layer chromatography (TLC) analyses and preparative TLC (pTLC) purification was performed on 250µm Silica Gel 60 F254 plates purchased from EM Science and Fluka Analytical. All TLC samples were stained using cerium ammonium molybdate stain (CAM stain) unless otherwise noted. All solvents and chemicals were used as purchased without further purification. Solvents used in the reactions were collected under nitrogen from a Pure Solv 400-5-MD Solvent Purification System (Innovative Technology).

NMR samples were prepared in 5 mm tubes with noted solvent. NMR data were all collected on a 300 MHz, 400 MHz, or 500 MHz Varian VNMRS DirectDrive spectrometer equipped with an indirect observe probe or 600 MHz 5 mm Nalaroc broad band probe. Chemical shifts for proton and carbon resonances are reported in ppm (δ) relative to the residual proton or the specified carbon in chloroform (δ 7.26, proton; 77.16, carbon). Data was collected under STP conditions. Pulse sequences were used as supplied by Varian VNMRJ 4.2 software. All 2D data employed non-uniform sampling (NUS). All data was processed in MestreNova v12.0.2

High-resolution mass spectrometry (HRMS) analysis was performed using Agilent 6538 high-mass-resolution QTOF mass spectrometer. HPLC purification was performed on Shimadzu LCMS 2020 system [LC-20AP (pump), SPD-M20A (diode array detector), LCMS-2020 (mass spectrometer)]. Semi-preparative HPLC purification was performed using Phenomenex Luna C-18(2) column, 5µm particle size (250 mm x 4.6 mm), supported by Phenomenex Security Guard cartridge kit C18 (4.0 mm x 3.0 mm); Phenomenex Luna C-8(2) column, 5µm particle size (250 mm x 4.6 mm), supported by Phenomenex Security Guard cartridge kit C8 (4.0 mm x 3.0 mm) and HPLC-grade solvents.

OSW-1 Compound

The OSW-1 compound used was obtained through total synthesis in the Burgett lab or from isolation from the natural source. OSW-1 used in the experiments was of >95% purity as determined through ^{1}H-NMR and LCMS analysis. Solid OSW-1 compound was dissolved in analytical grade DMSO solution to produce 10mM stocks for experimentation. The 10 mM OSW-1 stock solution was aliquoted into Eppendorf brand 1.5 mL centrifuge tubes; Each individual 10mM OSW-1 aliquots were thawed no more than three times.

T-00127-HEV2 (THEV, 3) Compound Synthesis

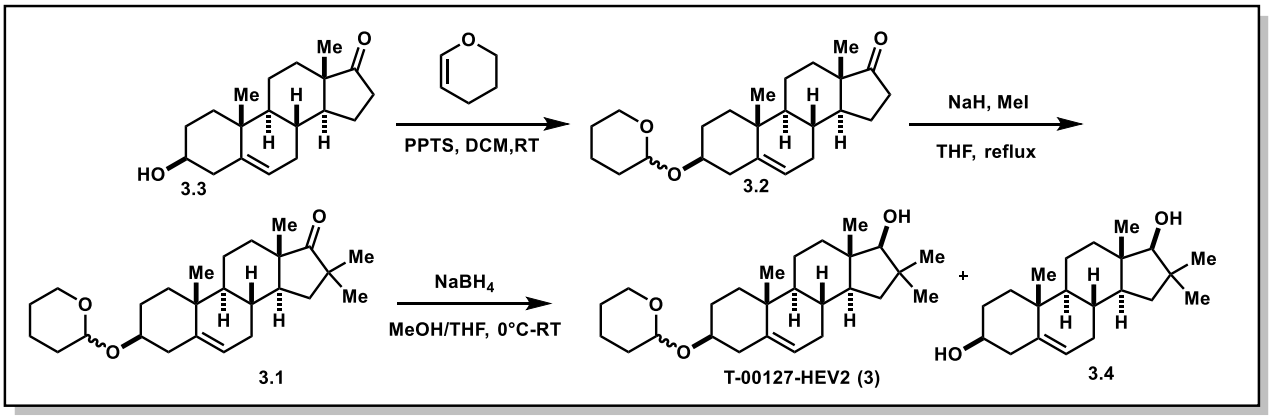


Figure 1. Synthesis of THEV (3) compound

Summary: T-00127-HEV-2 was produced through the following organic reaction steps. An experimental procedure and characterization data for the THEV compound used in previous published articles has not been reported to our knowledge. The intermediate compounds were carried forward as a mixture of mixture of diastereomers at the tetrahydropyran (THP) acetal position. The C17-hydroxyl stereochemistry is assigned via X-ray crystal analysis of the deprotected THEV compound (*vide infra*). The final T-00127-HEV2 compound was HPLC purified to separate the tetrahydropyran (THP) acetal diastereomers, and the major, unassigned THP diastereomer was used for all biological testing.

Compound **3.2**: THP protection of *trans*-dehydroandrosterone was performed as previously described.[5] To a solution of *trans*-dehydroandrosterone **3.3** (500 mg, 1.73 mmol) in anhydrous DCM (7 mL, 0.25 M) at RT under N_2 atmosphere was added 3,4-dihydro-2H-pyran (0.427 mL, 4.68 mmol) and pyridinium *p*-toluenesulfonate (21.8 mg, 0.086 mmol). The clear, colorless solution was stirred at RT, and the reaction progress was monitored by TLC (30% EtOAc/hexanes, UV, CAM stain). After 2 hours, the reaction was completed based on TLC. The reaction was quenched with DI H_2O (10 mL). The aqueous phase was extracted with DCM (5 mL x3). The combined organic phase was washed with brine, dried over Na_2SO_4. The solvent was removed to afford crude product as a white solid (656 mg, quantitative yield). ^1H and ^{13}C NMR showed desired product. This was taken to the next step without further purification. ^1H NMR (400 MHz, Chloroform-*d*) δ 5.43 – 5.34 (m, 1H), 4.71 (dt, *J* = 5.0, 2.7 Hz, 1H), 3.91 (ddt, *J* = 10.8, 5.6, 3.9

Hz, 1H), 3.61 – 3.41 (m, 2H), 2.45 (ddd, *J* = 19.1, 8.9, 1.0 Hz, 1H), 2.40 – 2.31 (m, 2H), 2.26 – 2.01 (m, 2H), 1.99 – 1.77 (m, 5H), 1.77 – 1.58 (m, 4H), 1.58 – 1.39 (m, 6H), 1.34 – 1.21 (m, 2H), 1.14 - 0.95 (m,2H), 1.03 (s, 3H), 0.88 (s, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 221.28, 141.49, 141.32, 120.94, 120.87, 97.07, 76.84, 76.00, 75.95, 63.03, 51.92, 51.91, 50.46, 50.43, 47.69, 40.37, 38.90, 37.53, 37.29, 37.09, 37.05, 35.99, 31.66, 31.59, 31.42, 30.96, 29.78, 28.09, 25.63, 22.03, 20.50, 20.18, 19.55, 13.69.

Compound **3.1**: Alpha methylation of compound **6** was performed as previously described.[6] To a solution of **3.2** (300 mg, 0.805 mmol) in anhydrous THF (5.03 mL, 0.16 M) at 0°C was added NaH (354 mg, 8.85 mmol) and iodomethane (0.5 mL, 8.06 mmol) fast dropwise. The gray, cloudy reaction mixture was stirred in the ice bath for 10 minutes, then at RT. The reaction progress was monitored by TLC (20% EtOAc/hexanes, UV, CAM stain). After 1 hour, the reaction mixture was heated under reflux. After 3.5 hours total, when TLC showed no further progress, the reaction was quench with EtOH (2 mL) at RT. The reaction mixture was then diluted with EtOAc (10 mL), washed with sat'd NH_4Cl solution. The aqueous phase was back extracted with EtOAc (5 mL x2). The combined organic phase was washed with DI H_2O, brine, and dried over Na_2SO_4. The solvent was removed to afford crude mixture as an off-white paste (411 mg). This mixture was separated by silica gel column chromatography (Biotage, 50g column, EtOAc/hex gradient from 2% to 30% in 10CV). 51.5mg of the desired C16-dimethylated product (**3.1**) was obtained in 16% yield. Additional mixed product fractions obtained but not carried forward. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.40 – 5.32 (m, 1H), 4.71 (dd, *J* = 5.2, 3.4 Hz, 1H), 3.96 – 3.86 (m, 1H), 3.57 – 3.45 (m, 2H), 2.42 – 2.31 (m, 1H), 2.25 – 2.16 (m, 0H), 2.13 – 2.00 (m, 1H), 1.95 – 1.78 (m, 4H), 1.78 – 1.68 (m, 2H), 1.67 – 1.60 (m, 3H), 1.59 – 1.43 (m, 5H), 1.43 – 1.33 (m, 1H), 1.28 (td, *J* = 13.0, 4.4 Hz, 1H), 1.18 (s, 3H), 1.13 – 1.04 (m, 1H), 1.03 (s, 3H), 1.02 (s, 3H), 0.90 (s, 3H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 225.44, 120.93, 97.05, 76.00, 62.99, 50.65, 48.73, 48.61, 45.32, 40.36, 38.91, 38.14, 37.51, 37.26, 37.14, 37.10, 36.90, 32.41, 31.42, 31.13, 29.78, 28.09, 27.44, 26.10, 25.63, 20.35, 20.19, 20.16, 19.54, 14.29

Compound **3 (T-00127-HEV-2)**: Reduction of compound **3.1** performed through using the previously developed procedure on a similar steroidal substrate.[7] To a solution of **3.1** (30.0 mg, 0.075 mmol) in anhydrous THF (0.75 mL, 0.1M) and MeOH (0.25 mL, 0.3M) at 0°C, $NaBH_4$ (22.7 mg, 0.60 mmol) was added as a solid. The reaction mixture was stirred at 0°C for 40 minutes, then at RT. The reaction progress was monitored by TLC (20% EtOAC/hex, UV, CAM stain). After 4 hours, the reaction was ~ 90% completed based on TLC. The reaction mixture was cooled to 0°C, and additional $NaBH_4$ (6.6 mg, 0.15 mmol) was added. The reaction mixture was stirred at 0°C for 10 minutes, then at RT for additional 2 hours to completion based on TLC. The reaction was quenched with acetone at RT. The reaction mixture was diluted in DI H_2O (10 mL), extracted with EtOAc (5 mL x3). The combined organic phase was washed with sat'd $NaHCO_3$ solution, brine, dried over Na_2SO_4. The solvent was removed to afford crude product as a clear gel (20.0 mg, 66%).

The crude products (diastereomeric mixture of the THP group, 20.0 mg) were separated through HPLC (C8 column, isocratic of 65% MeCN/0.1% formic acid in H_2O) to afford two separated C22-diastereomers, with a combined mass of 19.8 mg; 66% yield. Since the THP group is labile in acidic condition, due to the presence of minute amount of formic acid in the mobile phase of HPLC, a small amount of THP deprotected product was observed. The major C22-diastereomer was further purified through silica gel flash chromatography to afford desired

compound **3** (6.1 mg), along with the deprotected product (2.8 mg). No evidence of C17 diastereomers was observed and appears to be stereoselective due to the C19 methyl. The stereochemistry at the THP acetal of the purified THEV compound is not assigned. ^{1}H NMR (500 MHz, Chloroform-d) δ 5.38 – 5.32 (m, 1H), 4.72 (t, J = 3.9 Hz, 1H), 3.96 – 3.87 (m, 1H), 3.57 – 3.45 (m, 2H), 3.19 (d, J = 7.0 Hz, 1H), 2.40 – 2.30 (m, 2H), 1.96 (ddd, J = 12.4, 6.1, 4.1 Hz, 1H), 1.91 – 1.79 (m, 3H), 1.75 – 1.67 (m, 1H), 1.61 – 1.40 (m, 11H), 1.31 (d, J = 7.6 Hz, 1H), 1.28 – 1.20 (m, 1H), 1.14 (td, J = 12.8, 4.3 Hz, 1H), 1.04 (s, 3H), 1.01 (s, 3H), 1.00 (s, 3H), 0.99 – 0.94 (m, 1H), 0.77 (s, 3H). ^{13}C NMR (126 MHz, Chloroform-d) δ 141.32, 121.34, 96.99, 90.04, 76.08, 63.00, 50.55, 48.26, 45.11, 41.75, 40.39, 38.99, 38.12, 37.35, 37.05, 32.48, 31.87, 31.43, 31.26, 28.12, 25.65, 25.48, 20.64, 20.18, 19.57, 11.58.

Compound 3.4; Deprotected THEV: ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.35 (dt, *J* = 5.5, 1.9 Hz, 1H), 3.56 – 3.48 (m, 1H), 3.19 (d, *J* = 4.6 Hz, 1H), 2.30 (ddd, *J* = 13.0, 5.1, 2.3 Hz, 1H), 2.23 (ddt, *J* = 13.5, 10.3, 2.6 Hz, 1H), 1.99 – 1.92 (m, 1H), 1.89 – 1.79 (m, 3H), 1.62 – 1.52 (m, 3H), 1.52 – 1.41 (m, 4H), 1.24 (t, *J* = 12.7 Hz, 1H), 1.18 – 1.05 (m, 2H), 1.04 (s, 3H), 1.01 (s, 3H), 1.01 (s, 3H), 1.00 – 0.94 (m, 2H), 0.78 (s, 3H). ^{13}C NMR (126 MHz, , Chloroform-*d*) δ 140.99, 121.54, 90.01, 71.88, 50.51, 48.25, 45.10, 42.42, 41.74, 38.99, 38.11, 37.40, 36.77, 32.48, 31.82, 31.78, 31.25, 25.48, 20.65, 19.58, 11.58.

2D gCOSY was collected with 2 scans, 256 increments, a spectral width of 3633.7 Hz, an acquisition time of 0.2818 seconds and a relaxation delay of 1.0182 seconds. Data was zero-filled to 2048 data points with a gaussian fit of 20 Hz and sin bell in F1 then gaussian fit of 10 Hz and Cos^2 in F2 prior to Fourier Transform.

2D HSQCAD was collected with 8 scans, 256 increments, a spectral width of 3633.7Hz in f2 and 30165.9 Hz in f1, an acquisition time of 0.2818 seconds, a relaxation delay of 1.0182 seconds, and C-H coupling constant of 146 Hz. Data was zero-filled to 2048 data points with a gaussian fit of 20 Hz and sin bell 90 in F1 then gaussian fit of 7.64 Hz and Cos 90 in F2 prior to Fourier Transform.

2D HMBC was collected with 16 scans, 512 increments, a spectral width of 3633.7Hz in f2 and 30165.9 Hz in f1, an acquisition time of 0.5636 seconds, a relaxation delay of 0.7364 seconds. Data was not intensified using zero-filled or linear predict, a gaussian fit of 40 Hz and sin bell 90 in F1 then gaussian fit of 5 Hz and Cos 90 in F2 prior to Fourier Transform.

Crystallography data was collected of deprotected THEV (**3**). Upon HPLC purification, some of compound **3** underwent deprotection and the resulting C3 hydroxyl compound easily crystalized. Crystallography data of deprotected THEV (**3**) was collected and used in parallel with the NMR characterization to assign the C17 hydroxyl stereochemistry.

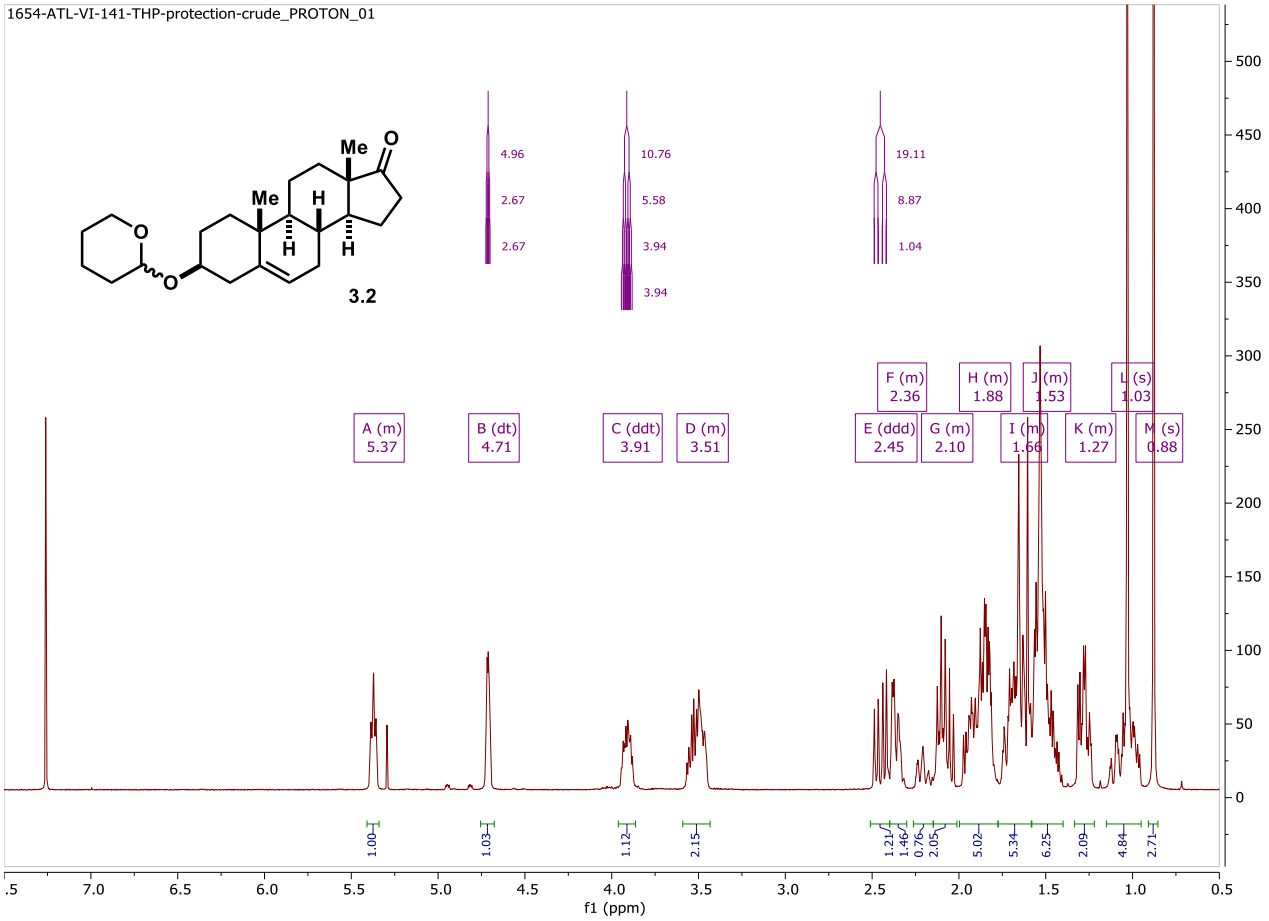


*Figure 2. [1]HNMR of **3.2** in d-chloroform*

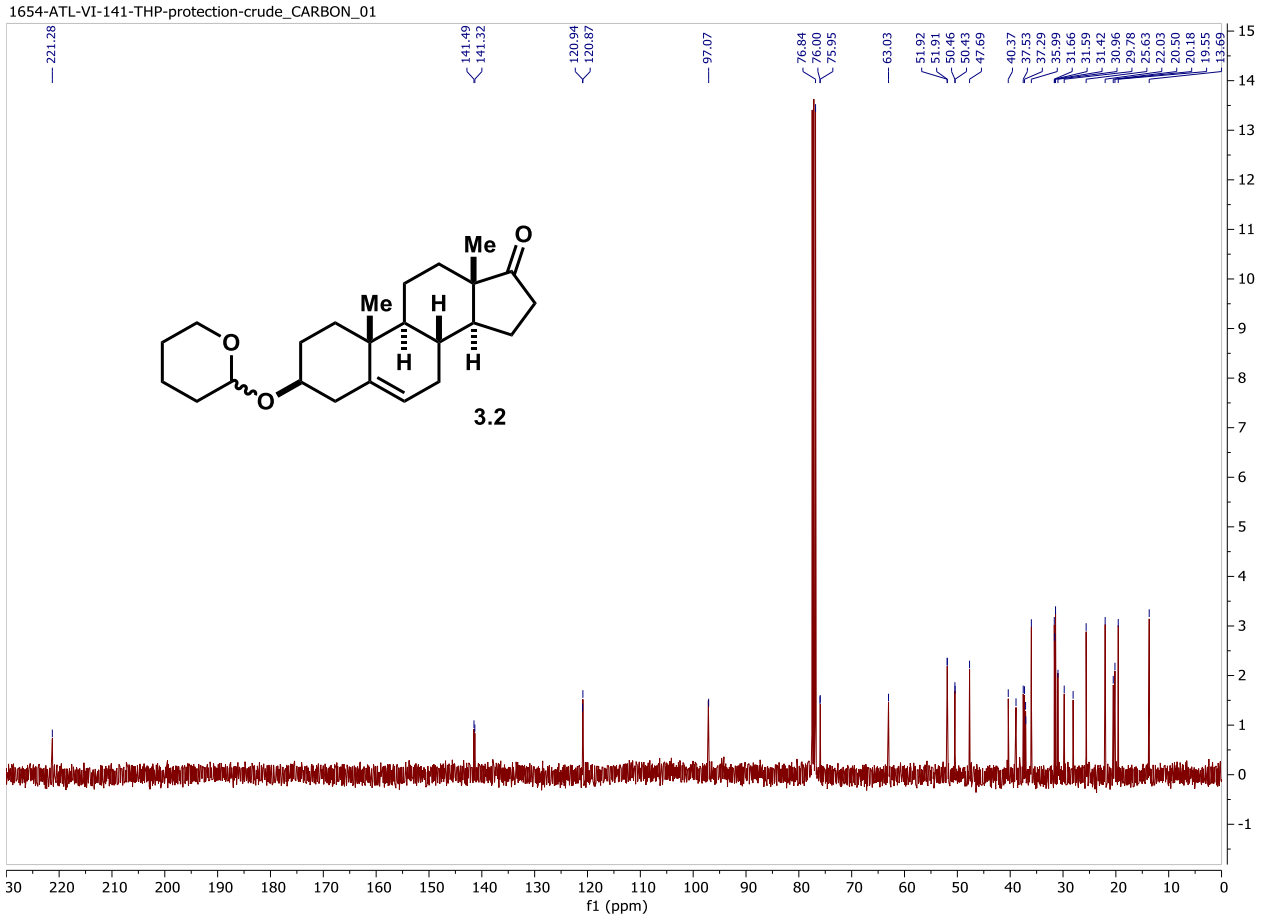


Figure 3. [13]CNMR of 3.2 in d-chloroform

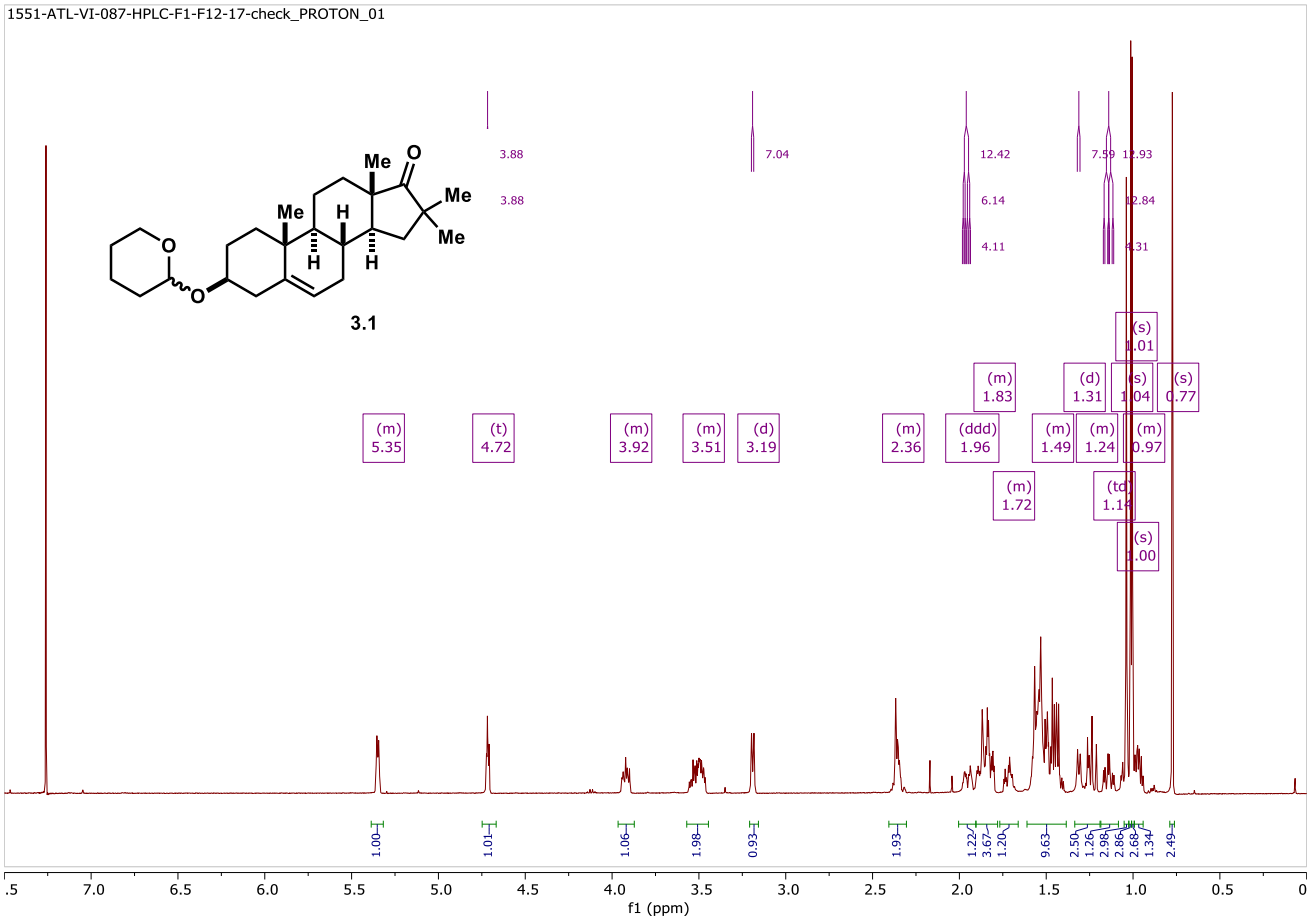


*Figure 4. [1]HNMR of **3.1** in d-chloroform*

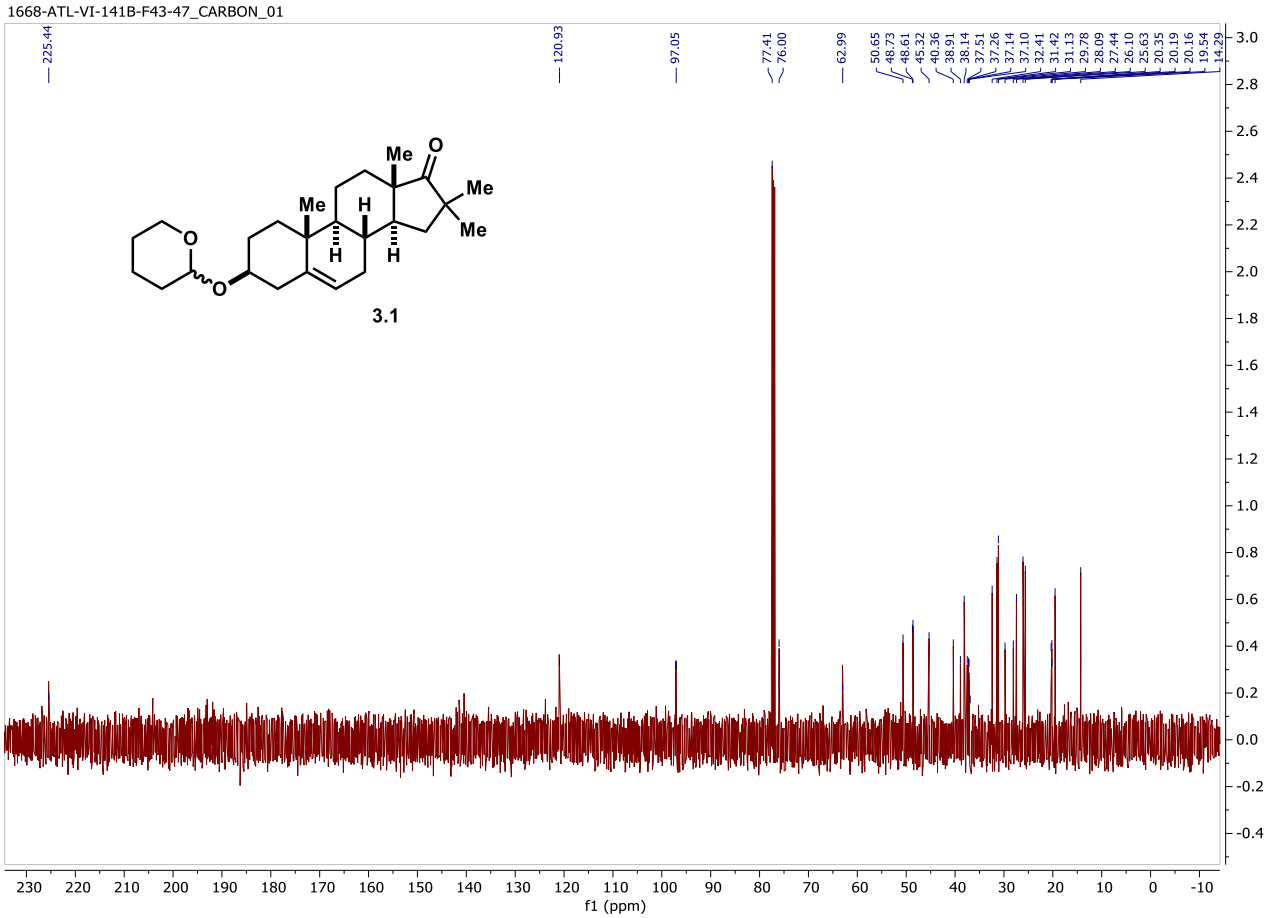


*Figure 5. CNMR of **3.1** in d-chloroform*

*Table 1. Spectrometric characterization of THEV (**3**)*

Atom	Chemical Shift	COSY	HSQC	HMBC
1 C	37.31		1', 1"	18
H'	1.04		1	
H"	1.84		1	3, 5, 9
2 C	28.05		2', 2"	4'
H'	1.44	3, 4'	2	9
H"	1.89	3, 4'	2	4
3 C	76.04		3	1", 4', 22
H	3.51	2', 2", 4'	3	
4 C	40.32		4'	2", 6
H'	2.35	2', 2", 3, 6, 7', 7"	4	2, 3, 5, 6, 10
H"		6		5, 10
5 C	141.31			1", 4', 4", 7', 7", 18
6 C	121.33		6	4', 7', 7"
H	5.34	4', 4", 7', 7"	6	4, 7, 8, 10
7 C	31.81		7', 7"	6
H'	1.97	4', 6	7	5, 6, 8, 9
H"	1.50	4', 6	7	5, 6, 8
8 C	31.19		8	6, 7', 7", 15', 15"
H	1.50		8	14
9 C	50.48		9	1", 2', 7', 18
H	0.97		9	
10 C	37.05			4', 4", 6
11 C	20.57		11', 11"	12'
H'	1.55		11	
H"	1.43		11	13
12 C	38.08		12', 12"	17, 19
H'	1.14		12	11, 13, 17, 19
H"	1.83		12	
13 C	45.12			11", 12', 15', 19
14 C	48.21		14	8, 15', 15", 19
H	0.98		14	
15 C	41.69		15', 15"	20, 21
H'	1.44		15	8, 13, 14, 16, 17, 20, 21
H"	1.24		15	8, 14, 16, 17, 20, 21
16 C	39.04			15', 15", 20, 21
17 C	89.99		17	12', 15', 15", 19
H	3.20	17'	17	12, 19, 20, 21
17' O H	1.33	17		
18 C	19.52		18	
H3	1.01		18	1, 5, 9
19 C	11.53		19	12', 17
H3	0.77		19	12, 13, 14, 17
20 C	32.43		20	15', 15", 17
H3	1.04		20	15, 16
21 C	32.43		21	15', 15", 17
H3	1.04		21	15, 16
22 C	96.95		22	26', 26"
H	4.71	23', 23"	22	3, 25, 26
23 C	31.36		23', 23"	
H'	1.55	22	23	25
H"	1.70	22	23	25
24 C	25.60		24'	25', 25"
H'	1.53		24	
H"				
25 C	20.13		25', 25"	22, 23', 23", 26', 26"
H'	1.84	26', 26"	25	24
H"	1.53	26', 26"	25	24
26 C	62.95		26', 26"	22
H'	3.47	25', 25", 26"	26	22, 25
H"	3.91	25', 25", 26'	26	22, 25

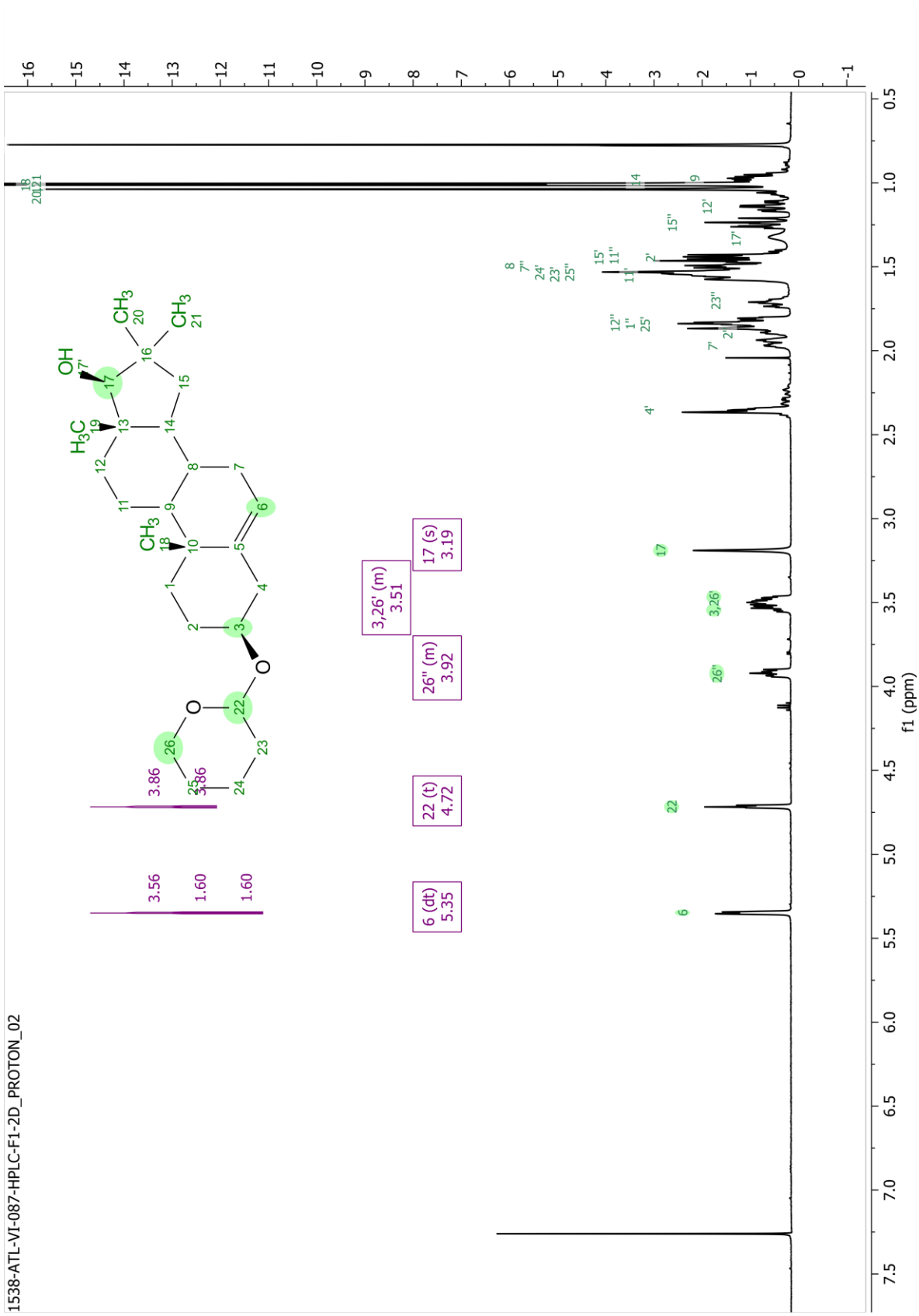


Figure 6. HNMR of T-00127-HEV2 (3) in d-chloroform

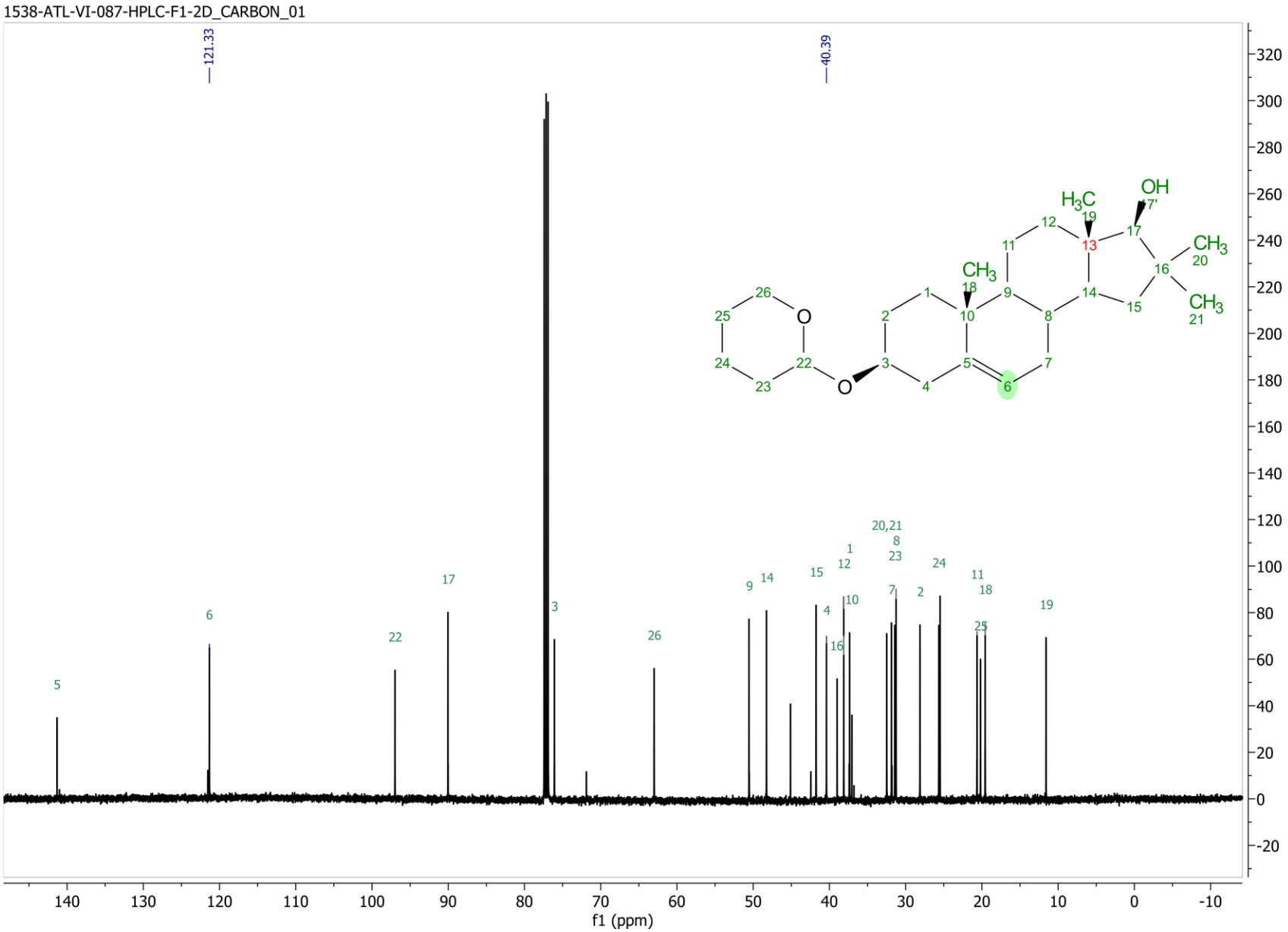


Figure 7. CNMR of T-00127-HEV2 (3) in d-chloroform

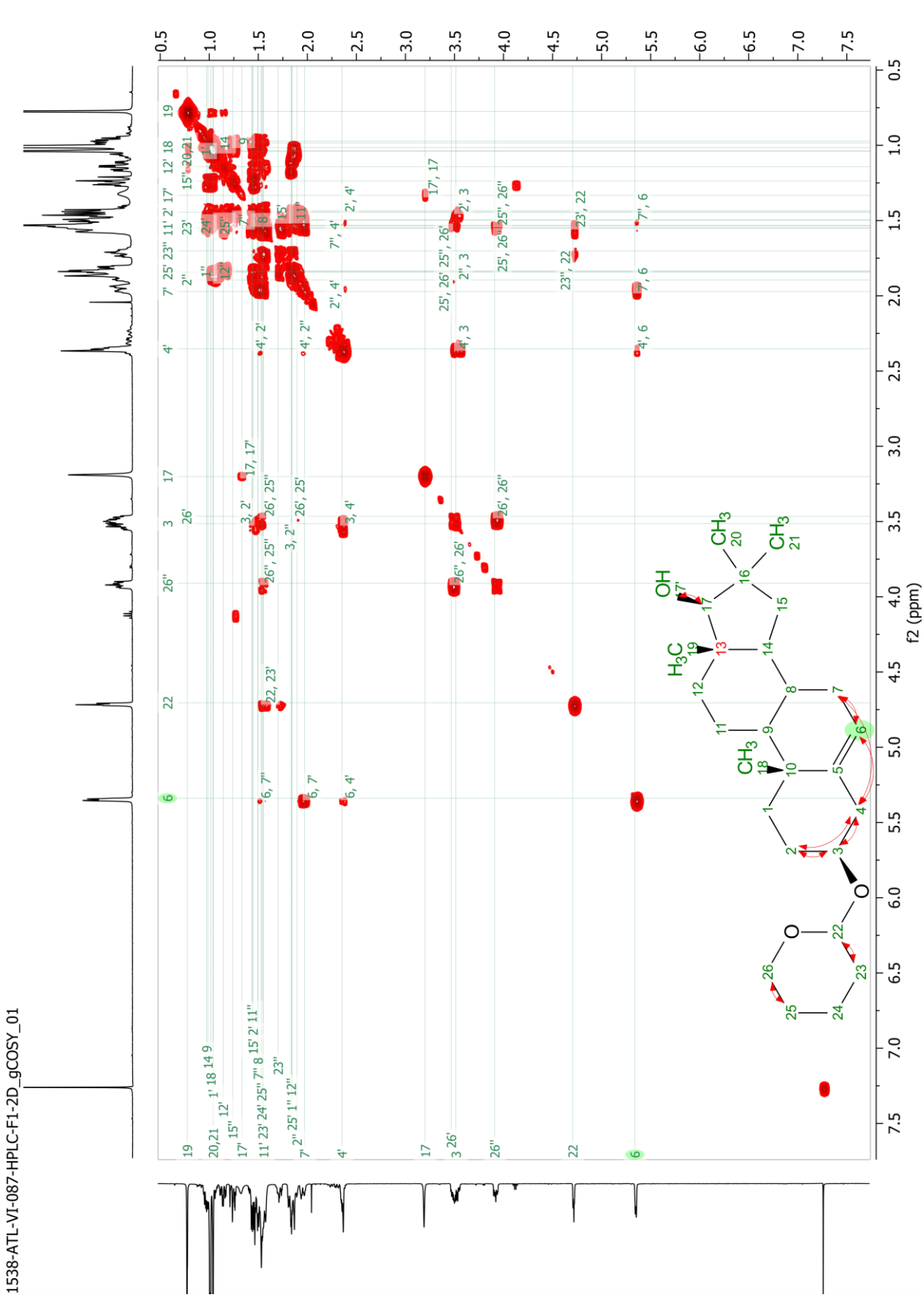


Figure 8. COSY of T-00127-HEV2 (3) in d-chloroform

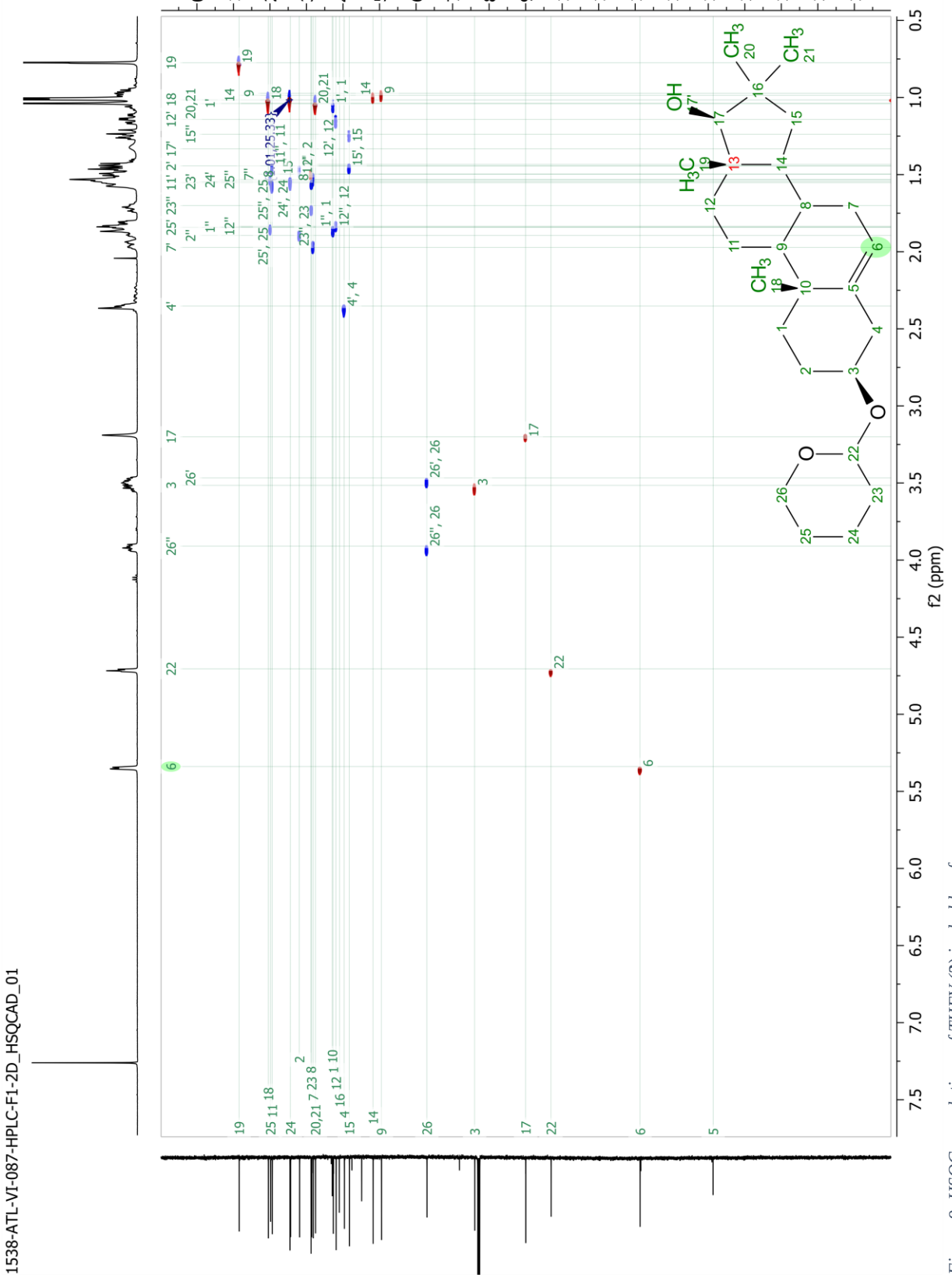


Figure 9. HSQC correlations of THEV (3) in d-chloroform

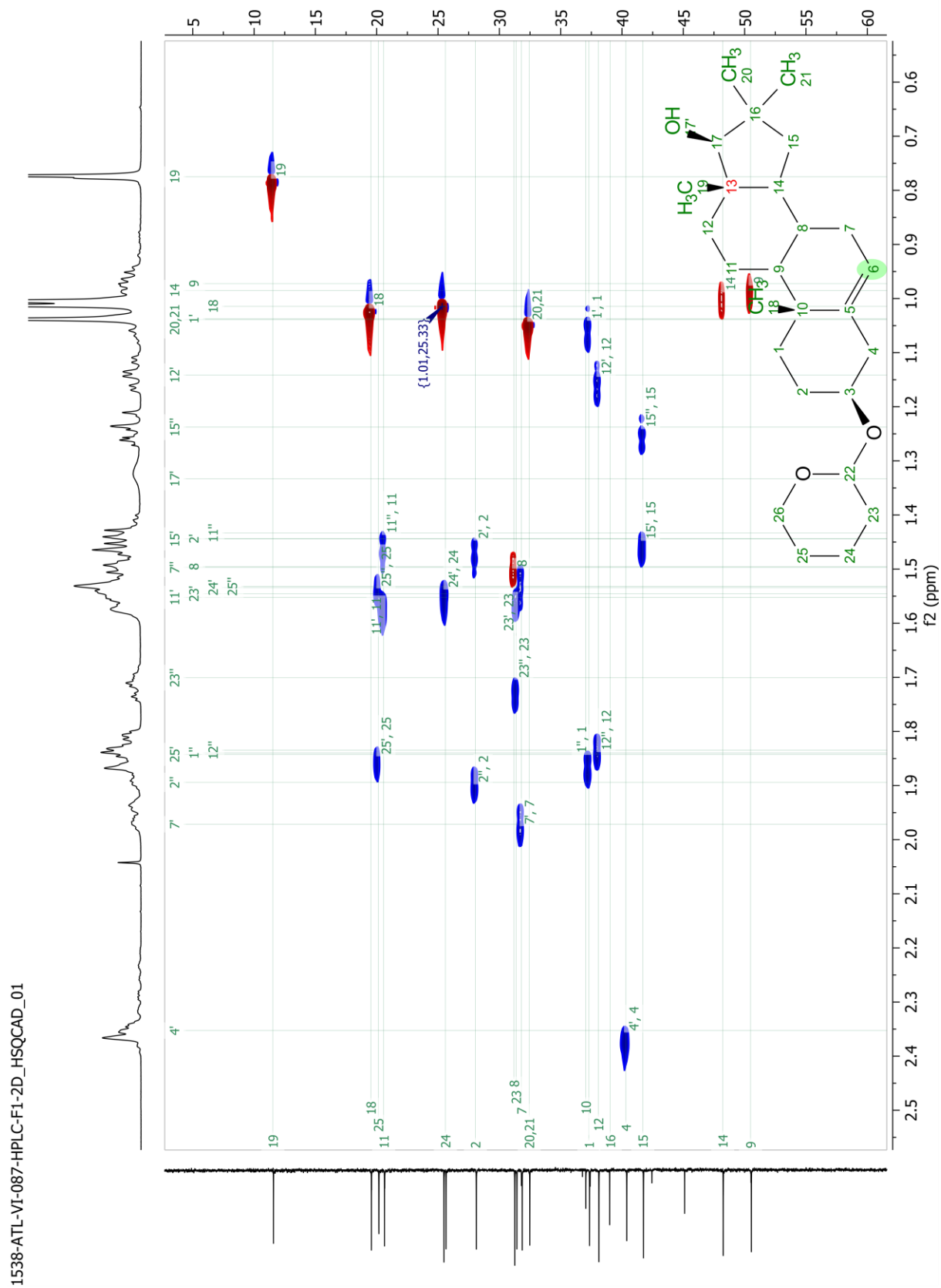


*Figure 10. HSQC (0.5-2.5ppm) of THEV (**3**) in d-chloroform*

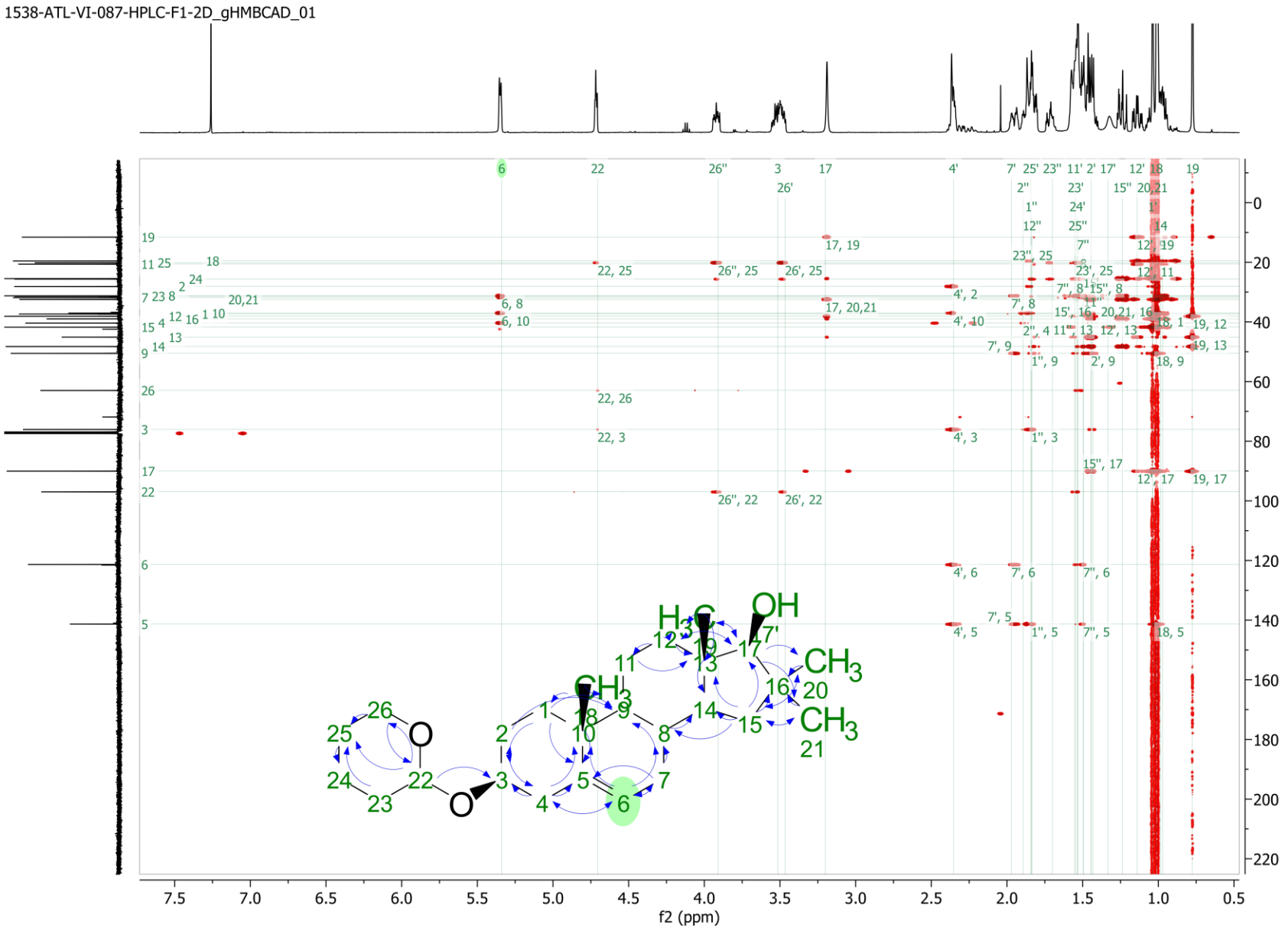


*Figure 11. HMBC correlations of THEV (**3**) in d-chloroform*

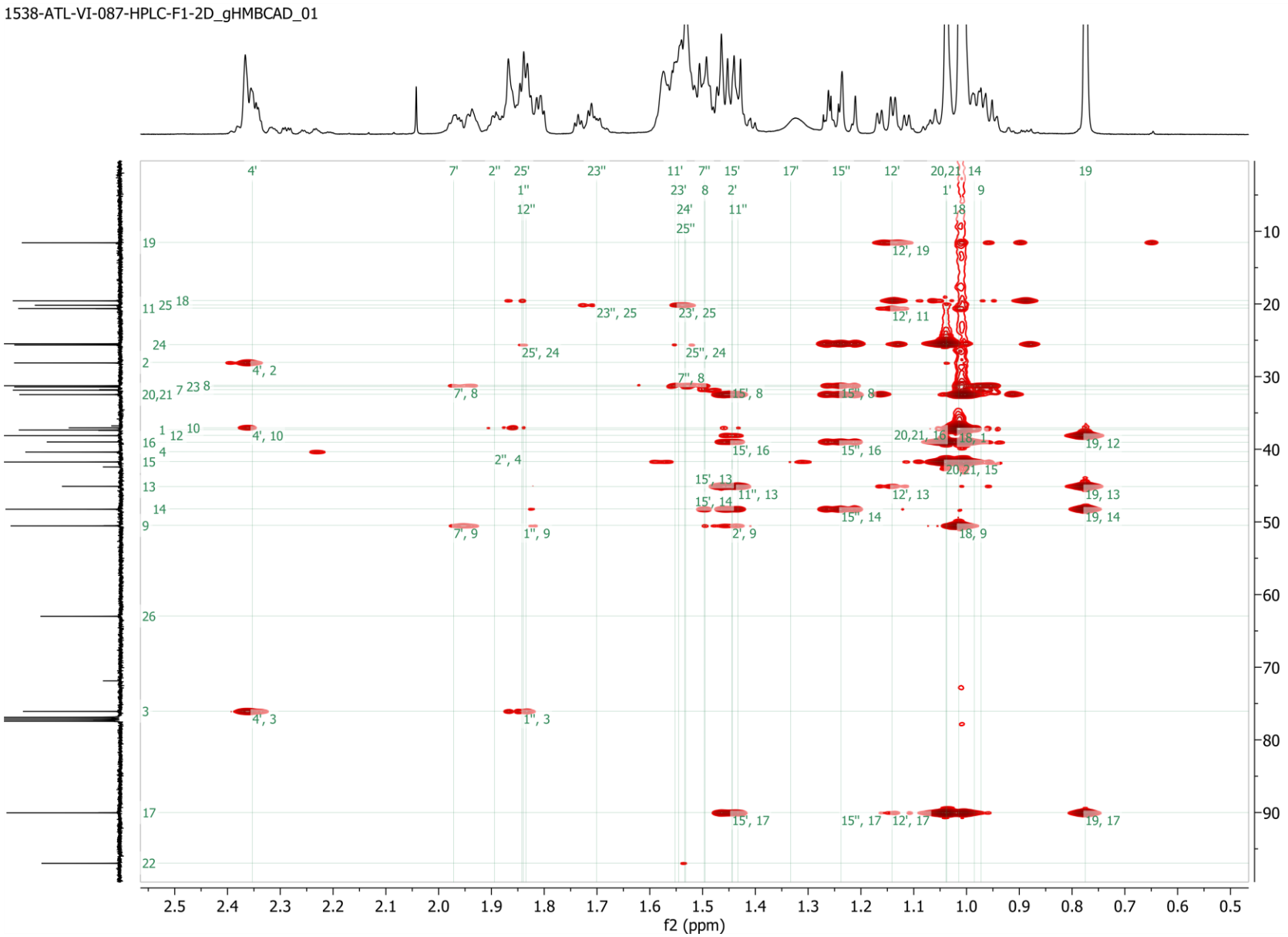


*Figure 12. HMBC (0.5-2.5 ppm) correlations for THEV (**3**) in d-chloroform*

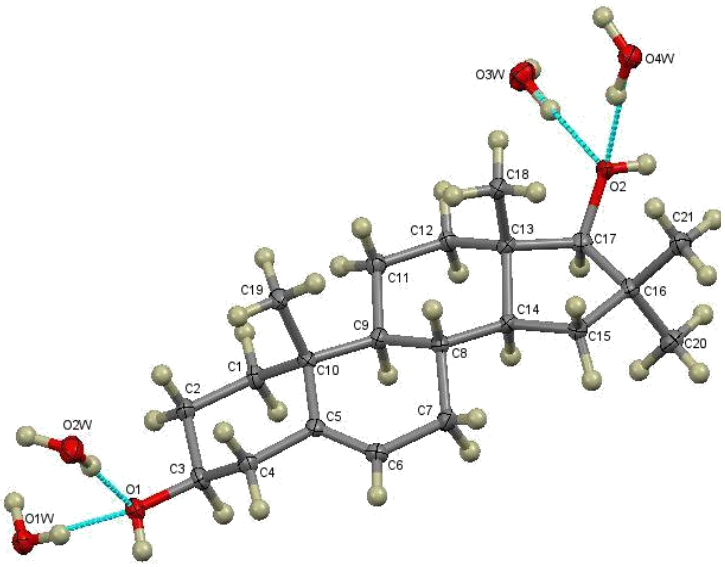


*Figure 13. X-Ray crystallography crystal of deprotected THEV (**3.4**)*

Crystallography Experimental

A colourless, needle-shaped crystal of dimensions 0.026 x 0.044 x 0.238 mm was selected for structural analysis. Intensity data for this compound were collected using a D8 Quest diffractometer with a Bruker Photon II ccd area detector and an Incoatec Iμs microfocus Mo Kα source (λ = 0.71069 Å). The sample was cooled to 100(2) K. Cell parameters were determined from a least-squares fit of 8128 peaks in the range $3.17 < \theta < 28.26°$. A total of 22144 data were measured in the range $2.173 < \theta < 28.431°$ using ϕ and ω oscillation frames. The data were corrected for absorption by the empirical method (2) giving minimum and maximum transmission factors of 0.6694 and 0.7457. The data were merged to form a set of 5472 independent data with R(int) = 0.0631 and a coverage of 99.9 %.

The monoclinic space group $P2_1$ was determined by systematic absences and statistical tests and verified by subsequent refinement. The structure was solved by direct methods and refined by full-matrix least-squares methods on F^2. The positions of hydrogens bonded to carbons were initially determined by geometry and were refined using a riding model. Hydrogens bonded to oxygens were located on a difference map, and their positions were refined independently with geometry restraints. Non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atom displacement parameters were set to 1.2 (1.5 for methyl) times the isotropic equivalent displacement parameters of the bonded atoms. A total of 280 parameters were refined against 36 restraints and 5472 data to give $wR(F^2) = 0.1318$ and S = 1.008 for weights of $w = 1/[\sigma^2 (F^2) + (0.0480\ P)^2 + 0.9800\ P]$, where $P = [F_o^2 + 2F_c^2] / 3$. The final R(*F*) was 0.0544 for the 4789 observed, [$F > 4\sigma(F)$], data. The largest shift/s.u. was 0.004 in the final refinement cycle. The final difference map had maxima and minima of 0.224 and -0.273 e/Å^3, respectively. The absolute structure was verified by refinement of the Hooft parameter.

*Table 2. Crystal data and structure refinement for deprotected THEV (**3.4**)*

Empirical formula	$(C_{21}H_{34}O_2) \cdot 4(H_2O)$	
	$C_{21}H_{42}O_6$	
Formula weight	390.54	
Crystal system	monoclinic	
Space group	*P*2**1**	
Unit cell dimensions	*a* = 6.0525(9) Å	α= 90°
	b = 11.8559(18) Å	β= 90.153(6)°
	c = 15.292(3) Å	γ= 90°
Volume	1097.3(3) $Å^3$	
Z, Z'	2, 1	
Density (calculated)	1.182 Mg/m^3	
Wavelength	0.71069 Å	
Temperature	100(2) K	
F(000)	432	
Absorption coefficient	0.084 mm^{-1}	
Absorption correction	semi-empirical from equivalents	
Max. and min. transmission	0.7457 and 0.6694	
Theta range for data collection	2.173 to 28.431°	
Reflections collected	22144	
Independent reflections	5472 [R(int) = 0.0631]	
Data / restraints / parameters	5472 / 36 / 280	
wR(F^2 all data)	*wR*2 = 0.1318	
R(*F* obsd data)	*R*1 = 0.0544	
Goodness-of-fit on F^2	1.008	
Observed data [I > 2□(I)]	4789	
Absolute structure parameter	-0.4(6)	
Extinction coefficient	0.051(7)	
Largest and mean shift / s.u.	0.004and 0.000	
Largest diff. peak and hole	0.224 and -0.273 e/$Å^3$	

*Table 3. Spectrometric characterization of deprotected THEV (**3.4**)*

Atom	Chemical Shift	COSY	HSQC	HMBC
1 C	37.39		1'	19
H'	1.08		1	2, 3, 10, 19
H"	1.86			2, 3, 10, 19
2 C	31.79		2', 2"	1', 1"
H'	1.85	3, 4'	2	3, 5, 10
H"	1.51	3, 4'	2	3, 5, 10
3 C	71.89		3	1', 1", 2', 2", 4', 4"
H	3.52	2', 2", 4', 4"	3	
4 C	42.42		4', 4"	6
H'	2.29	2', 2", 3, 6, 7', 7"	4	3, 5, 6, 7, 10
H"	2.24	3, 6	4	3, 6, 7, 10
5 C	140.99			2', 2", 4', 7', 19
6 C	121.54		6	4', 4", 7', 7"
H	5.35	4', 4", 7', 7"	6	4, 7, 8, 10
7 C	31.83		7', 7"	4', 4", 6
H'	1.98	4', 6, 7"	7	5, 6, 8, 9
H"	1.51	4', 6, 7'	7	6, 9
8 C	31.24			6, 7', 9, 11', 15"
H	1.50			
9 C	50.51		9	7', 7", 11', 11", 12', 12", 19
H	0.98		9	8, 11, 19
10 C	36.77			1', 1", 2', 2", 4', 4", 6, 19
11 C	20.65		11', 11"	9, 12', 12"
H'	1.57		11	8, 9, 13
H"	1.46		11	9, 12, 13
12 C	38.10		12', 12"	11", 17, 18
H'	1.84		12	9, 11, 13, 18
H"	1.14		12	9, 11, 13, 17, 18
13 C	45.10			11', 11", 12', 12", 15', 18
14 C	48.29		14	15', 15", 18
H	0.99		14	18
15 C	41.75		15', 15"	
H'	1.45		15	13, 14, 16, 17
H"	1.24		15	8, 14, 16, 17, 21
16 C	38.99			15', 15", 20
17 C	90.02		17	12", 15', 15", 18
H	3.19		17	12, 18, 20
18 C	11.60		18	12', 12", 14, 17
H3	0.78		18	12, 13, 14, 17
19 C	19.59		19	1', 1", 9
H3	1.02		19	1, 5, 9, 10
20 C	32.49		20	17
H3	1.04		20	16, 21
21 C	25.48		21	15", 20
H3	1.01		21	

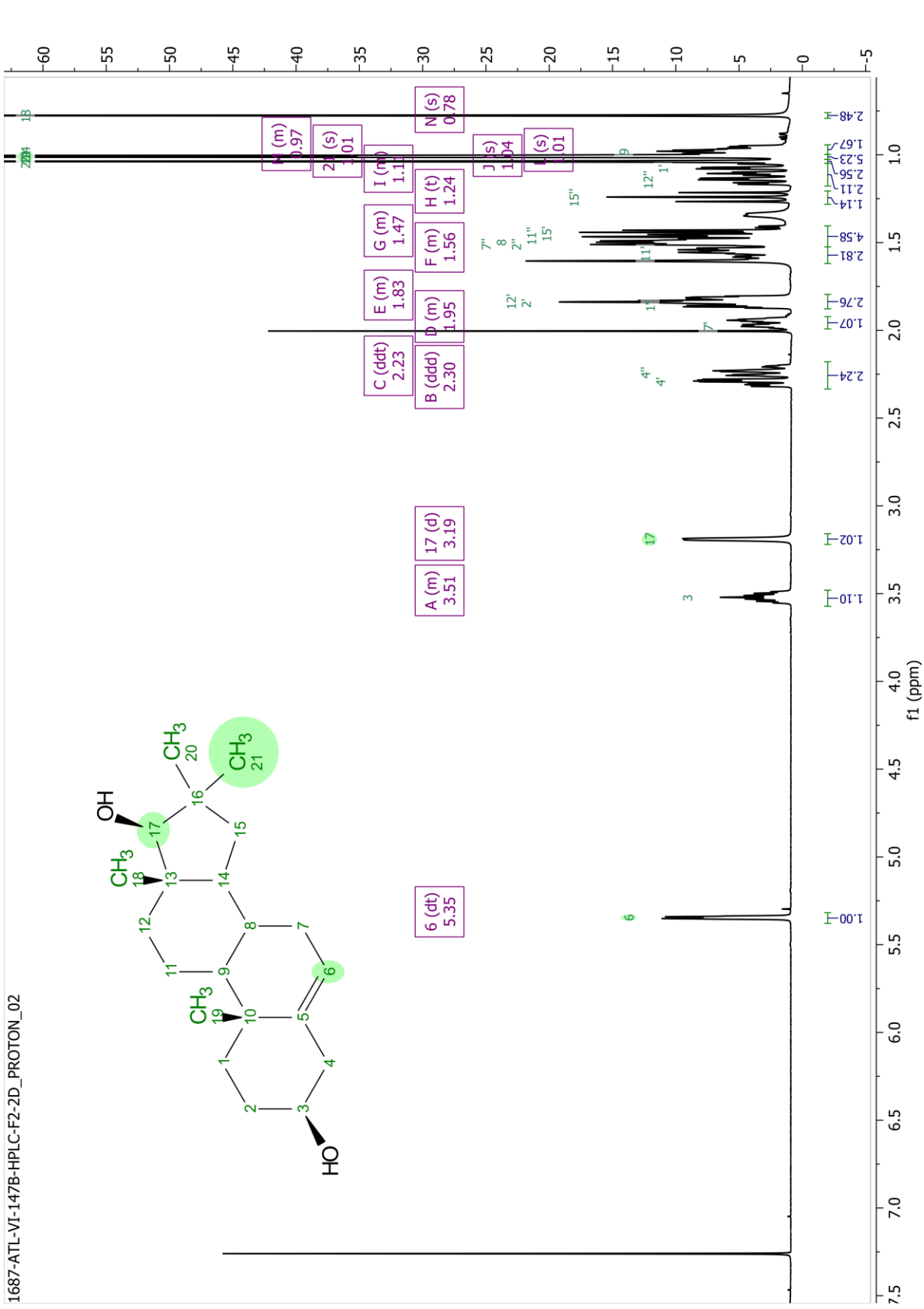


*Figure 14. ^{1}HNMR of deprotected THEV (**3.4**) in d-chloroform*

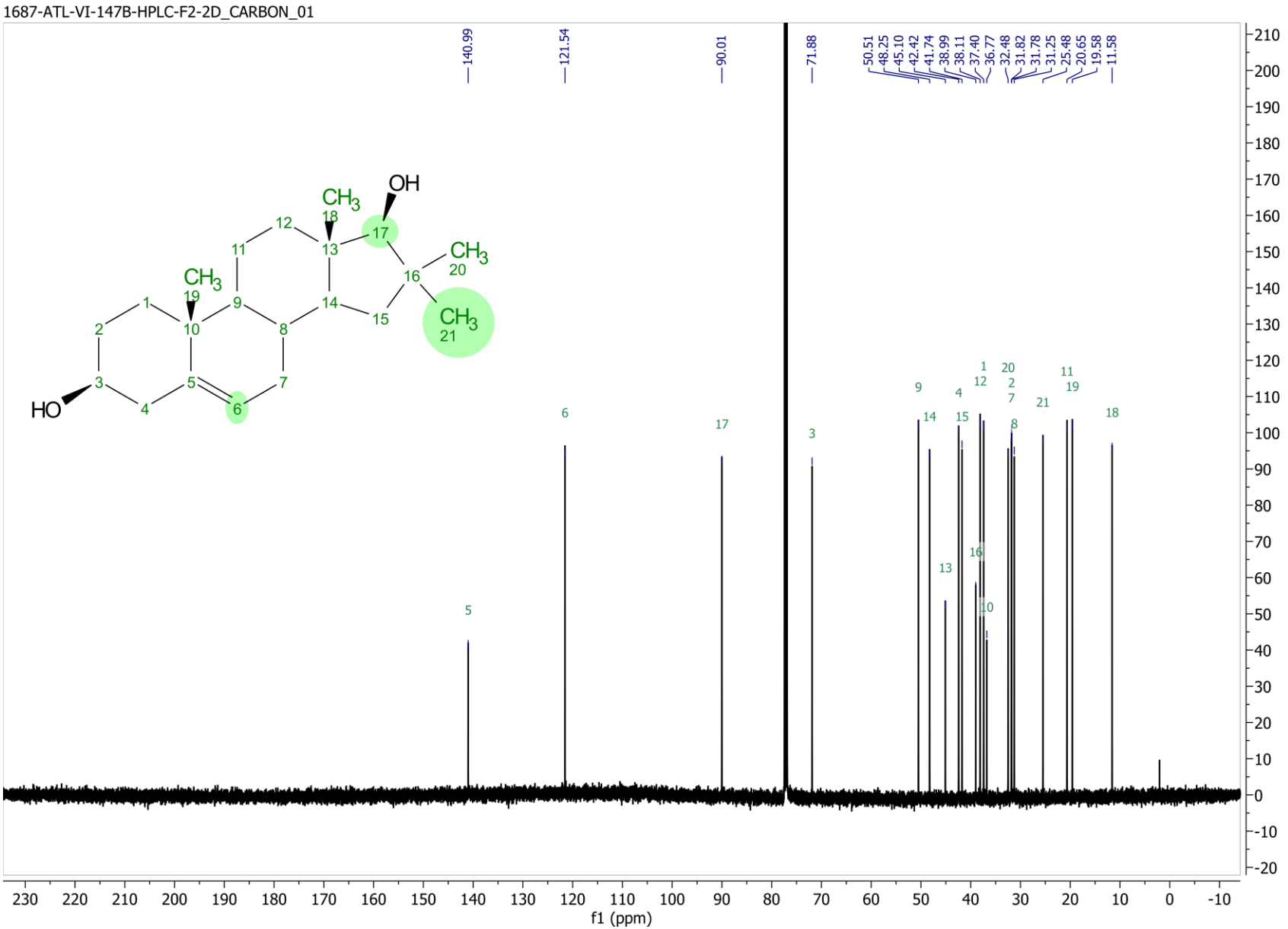


*Figure 15. ^{13}CNMR of deprotected THEV (**3.4**) in d-chloroform*

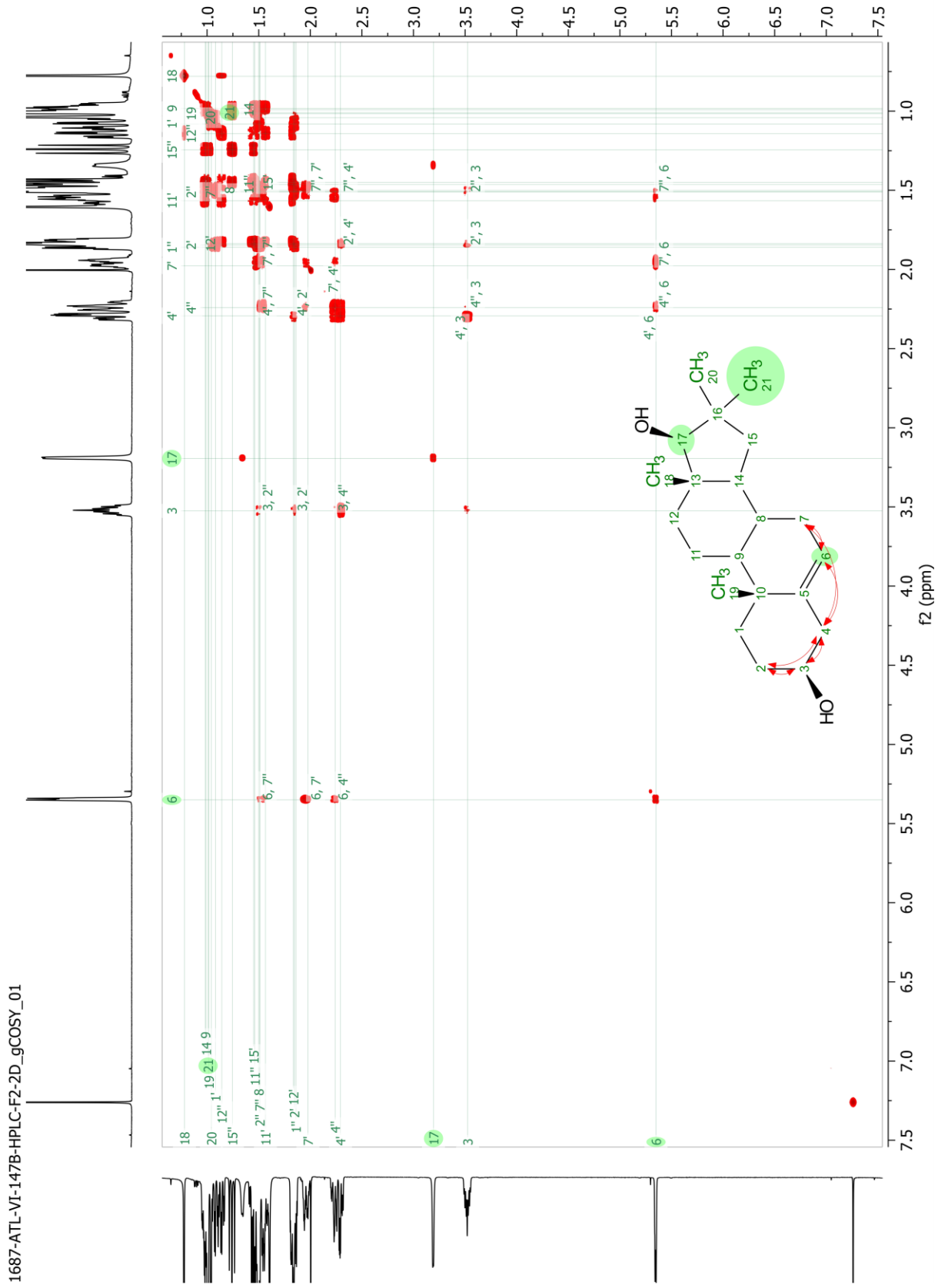


*Figure 16. COSY correlations of deprotected THEV (**3.4**) in d-chloroform*

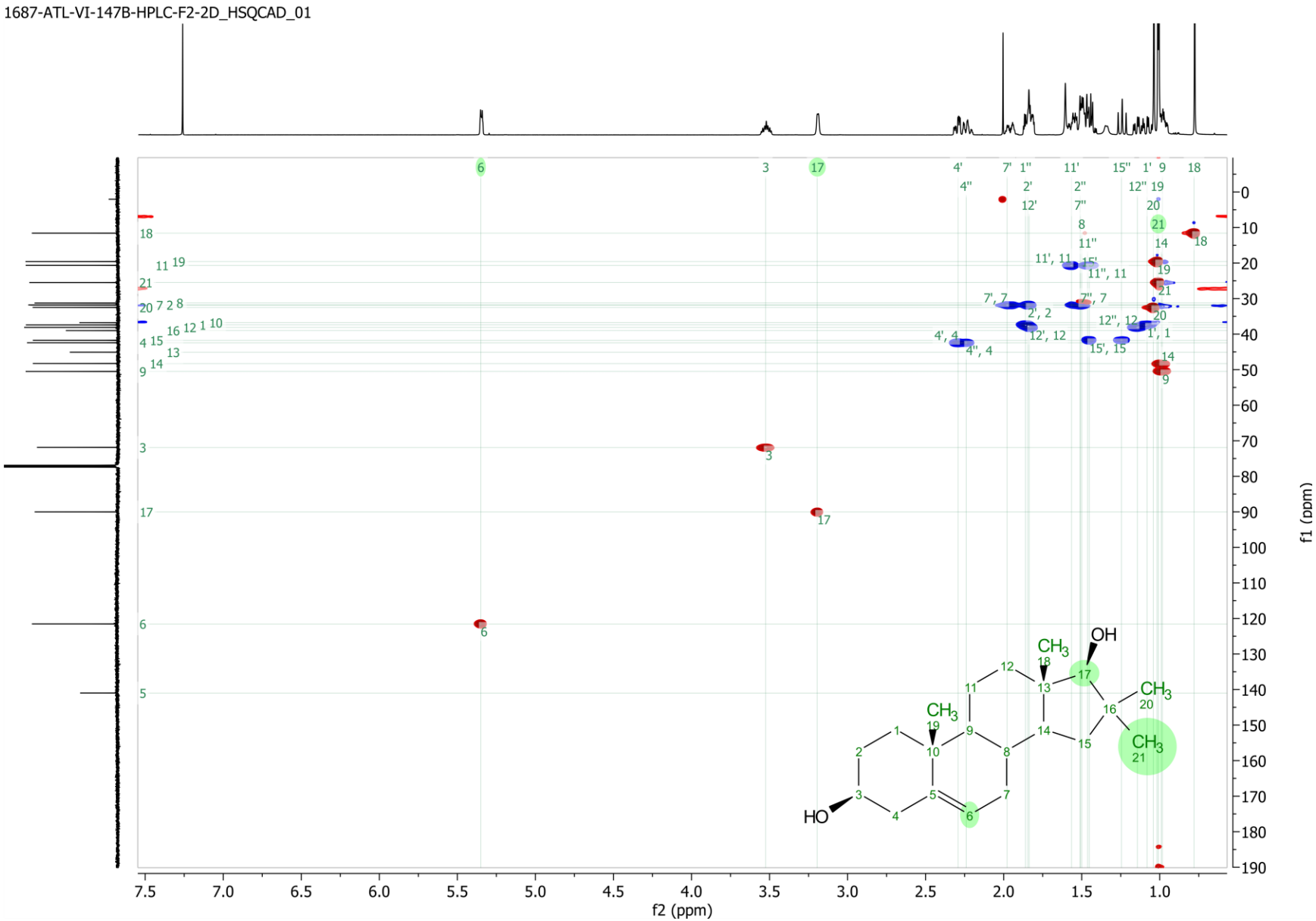
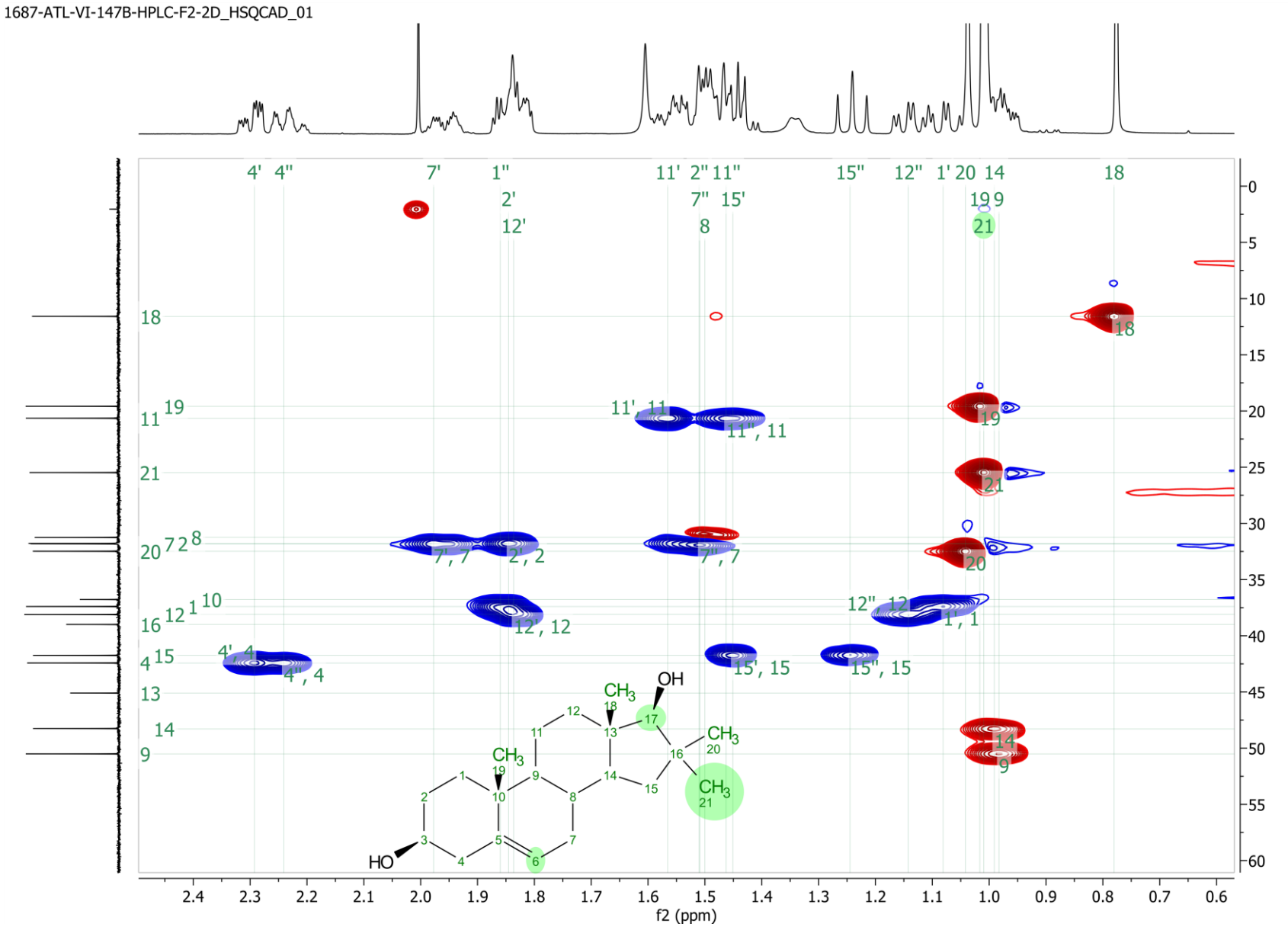


*Figure 17. HSQC correlations of deprotected THEV (**3.4**) in d-chloroform*


1687-ATL-VI-147B-HPLC-F2-2D_HSQCAD_01
f2 (ppm)
HO
OH
CH3

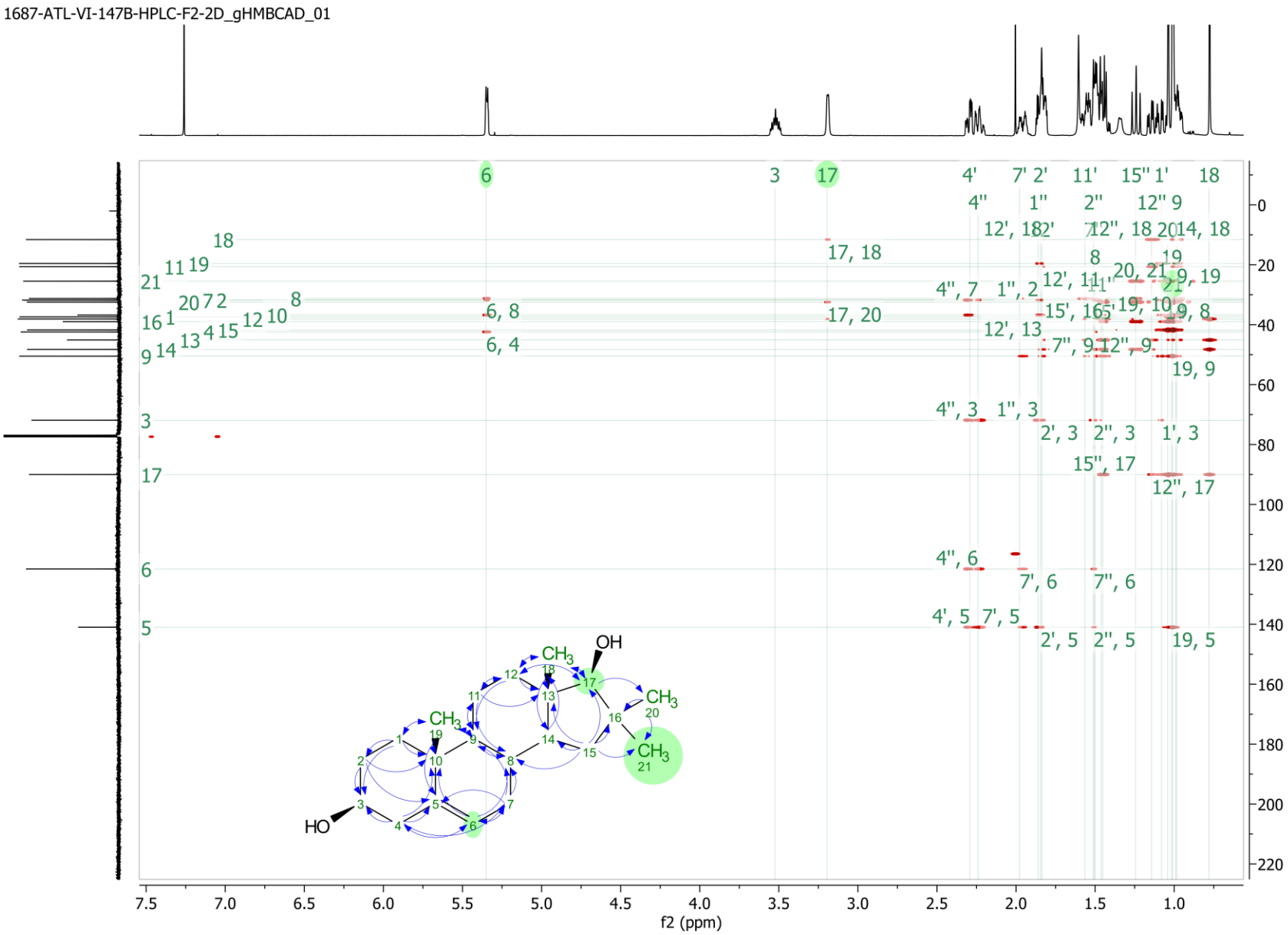


*Figure 18. HMBC correlations of deprotected THEV (**3.4**) in d-chloroform*

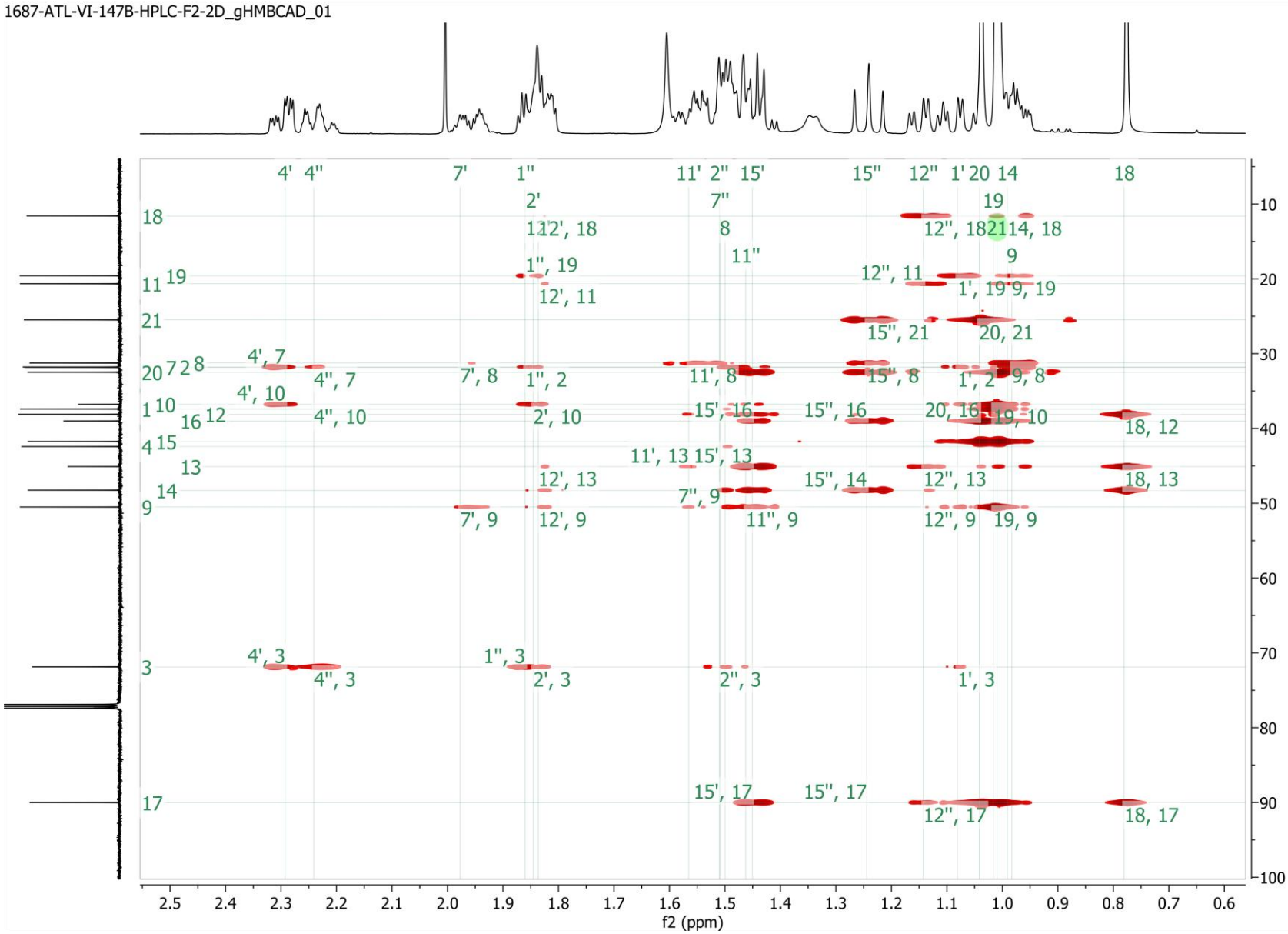


*Figure 19. HMBC correlations between 0.5-2.5 ppm of deprotected THEV (**3.4**) in d-chloroform*

TTP-8307 (5) Compound Synthesis

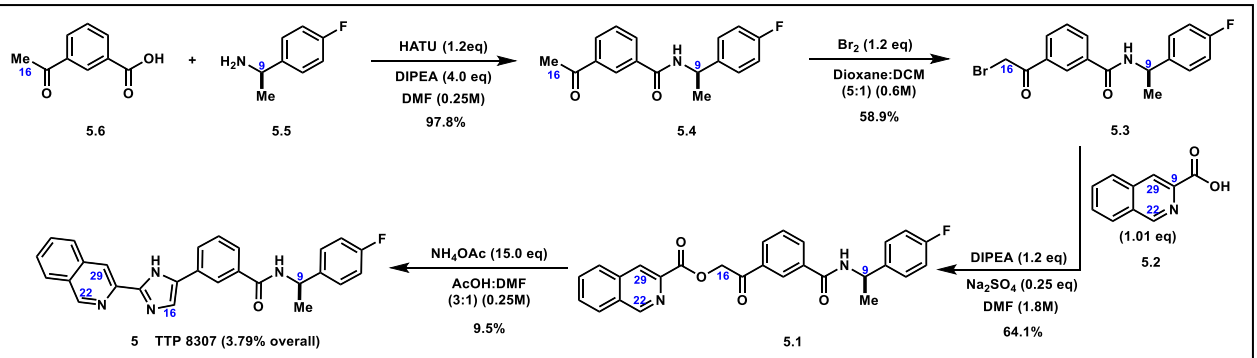


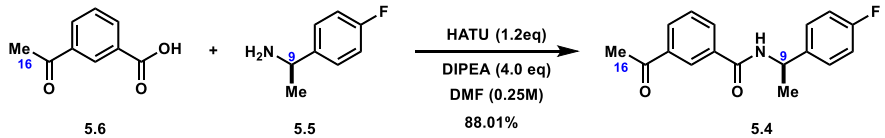
*Figure 20. Synthesis of TTP (**5**) compound*

A four-step linear synthesis of TTP-8307 (TTP, **5**) was carried out as previously described.[8] Beginning with coupling 3-acetylbenzoic acid (**5.6**) to 4-fluoro methyl benzyl amine (**5.5**) using HATU coupling agent and diisopropyl ethyl amine (DIPEA), the corresponding amide (**5.4**) was afforded. Amide **5.4** then underwent α-bromination at the acetyl position to produce bromo amide (**5.3**) which was then coupled to isoquinoline-3-carboxylic acid (**5.2**) via base mediated nucleophilic substitution. The terminating step formed the imidazole ring through two subsequent imine formations to eliminate water upon cyclization and arrive at title compound, TTP (**5**). Although ^{1}HNMR and ^{13}CNMR have previously been reported[8] for TTP, we performed HRMS and 2D NMR characterization methods.

All 2D gCOSY was collected with 8 scans, 512 increments, a spectral width of 5367.7 Hz, an acquisition time of 0.1908 seconds and a relaxation delay of 1.0 seconds. Data was not intensified using zero-filled or linear predict, sin square II applied in both F1 and F2 prior to Fourier Transform.

All 2D HSQCAD was collected with 8 scans, 256 increments, a spectral width of 5000.0 Hz in f2 and 17597.9 Hz in f1, an acquisition time of 0.2048 seconds, a relaxation delay of 1.0952 seconds, and C-H coupling constant of 146 Hz. Data was not intensified using zero-filled or linear predict, sin square II applied in both F1 and F2 prior to Fourier Transform.

CAM-III-33-HATU Coupling



Reactants						*Products*	
Formula	$C_9H_8O_3$	$C_8H_{10}FN$	$C_8H_{19}N$	$C_{10}H_{15}F_6N_6OP$	C_3H_7NO	Formula	$C_{17}H_{16}FNO_2$
MW	164.16	139.17	129.25	380.24	73.10	MW	285.32
Limiting?	***Yes***	No	No	No	No	Equivalents	
Equivalents	***1.00***	***1.00***	***4.00***	***1.20***		%Completion	
Sample Mass	***1.00g***	847.79mg	3.15g	2.78g	445.27mg	Expected Mass	1.74g
%Weight						Expected Moles	6.09mmol
Molarity	***250.00mM***					Measured Mass	***1.53g***
Density		***1.03g/mL***	***742.00mg/mL***			Purity	
Volume	24.37mL	823.10mmL	4.24mL			Product Mass	1.53g
Reactant Moles	6.09mmol	6.09mmol	24.37mmol	7.31mmol	6.09mmol	Product Moles	5.36mmol
Reactant Mass	1.00g	847.79mg	3.15g	2.78g	445.27mg	%Yield	88.03%

HATU Coupling to (R)-3-acetyl-N-(1-(4-fluorophenyl)ethyl)benzamide (**5.4**): To a 25 mL conical flask equipped with spin vane was added solid 2-acetyl benzoic acid (1.00 g, 6.09 mmol). The flask was septa sealed and then thrice purged using house nitrogen and vacuum. Dimethyl formamide (DMF, 2.0 mL) from sure-seal bottle was added to flask to dissolve starting material. In a 4mL dram vial, HATU coupling agent (277.95 mg, 7.31 mmol) was weighed out then dissolved in DMF (4.0 mL) while open to the environment. Dissolved HATU was added fast drop-wise via syringe followed by nitrogen purge of the flasks. Diisopropyl ethyl amine (DIPEA, 425 µl, 2.44 mmol) added slow drop-wise to the bulk over 15 minutes. Reaction turned yellow and progressively became a dark brown, over 10 min. 4-fluoro methyl benzyl amine (**5.5**, 82.31 µL, 6.09 mmol) was added to the bulk via syringe drop-wise in, no color change was observed. Reaction proceeded over 2 hours and monitored via TLC with 15% ethyl acetate in hexanes as the mobile phase. Product was UV active and imaged via $KMnO_4$ stain (CAM stain also imaged), presented with 0.5 Rf. Upon complete conversion as seen by TLC, reaction was diluted with distilled water, and back extracted four times using ethyl acetate. The organic layer was washed with brine twice then dried over sodium sulfate. Drying agent filtered off and crude material was condensed *in vacuo* to produce 1.54g (88.03% yield) of crude amide **5.4** as a white solid which was confirmed via ^{1}HNMR (**Figure 21**) and HRMS (**Figure 22**). ^{1}HNMR showed residual ethyl acetate and DMF solvents. Material pushed forward without further purification.

^{1}H NMR (500 MHz, Chloroform-*d*) δ 1.61 (d, *J* = 6.96 Hz, 3H, 10), 2.64 (s, 3H, 16), 5.32 (p, *J* = 7.09 Hz, 1H, 9), 6.57 (s, 1H, 8), 7.04 (t, *J* = 8.68 Hz, 2H, 13, 13'), 7.37 (t, *J* = 8.58, 6.66 Hz, 2H, 12, 12'), 7.54 (t, *J* = 7.76 Hz, 1H, 2), 8.02 (dt, *J* = 5.80, 1.44 Hz, 1H, 1), 8.07 (dt, *J* = 7.83, 1.43 Hz, 1H, 3), 8.33 (s, 1H, 5). Residual DMF peaks at: 2.87 (dt, *J* = 7.79, 3H), 2.95 (s, 2H, 4), 8.02 (s, 1H), Residual EtOAc peaks at: 1.25 (td, *J* = 7.14, 0.77 Hz, 1H), 2.04 (s, 1H, 3), 4.11 (q, *J* = 7.12 Hz, 1H)

HRMS (-ESI): m/z calculated $C_{17}H_{16}FNO_2$ -H+ [M - H+]: 284.1092 m/z, observed 284.1089 m/z, Δ = 1.055932 ppm

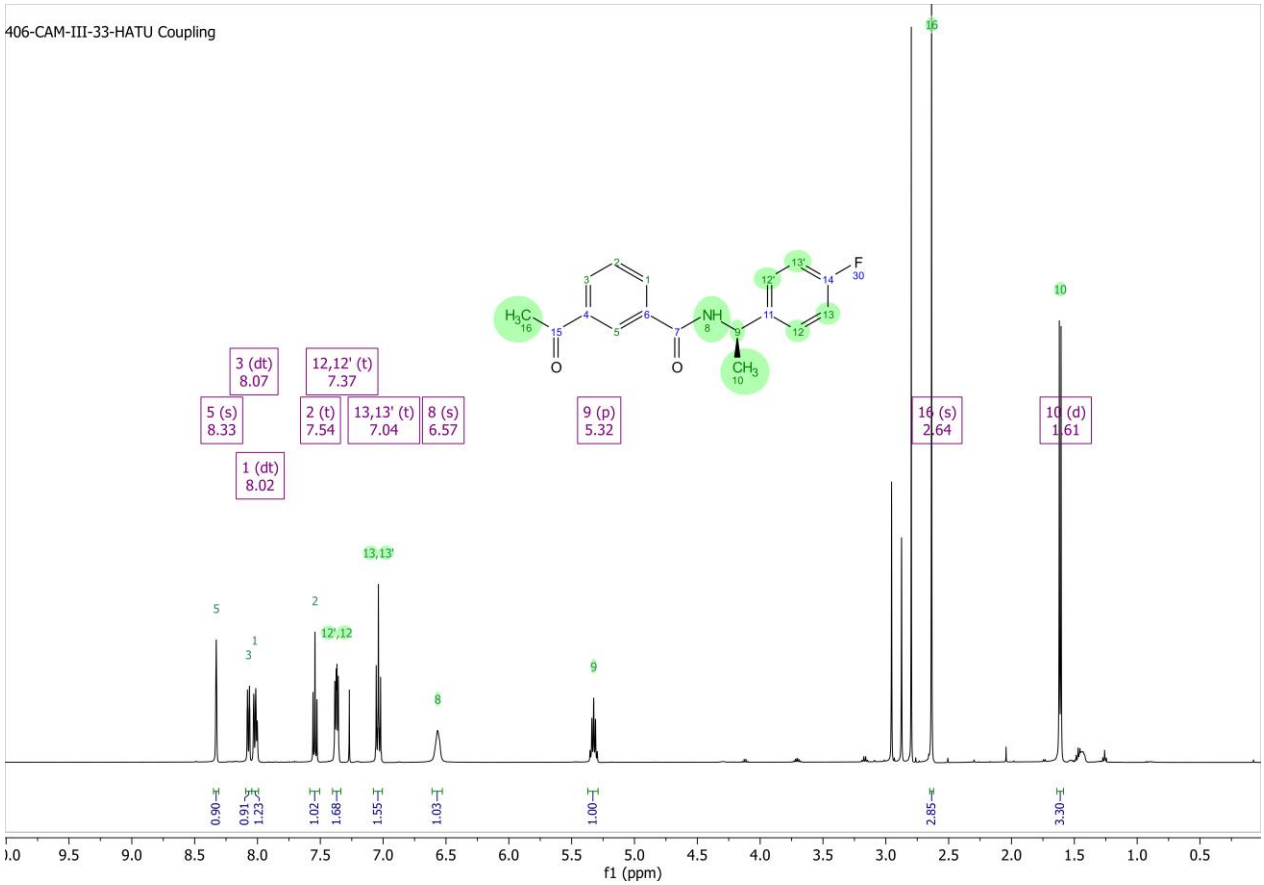


Figure 21. [1]HNMR of amide 5.4 in d-chloroform

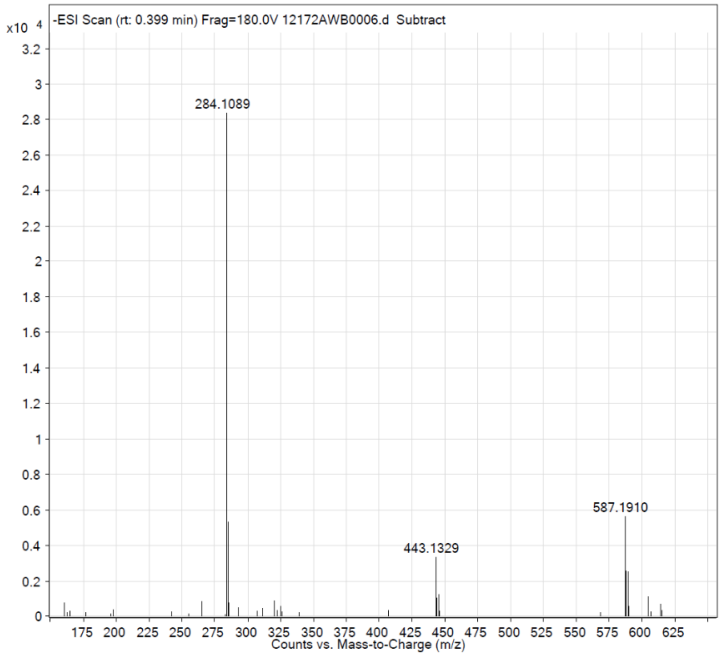
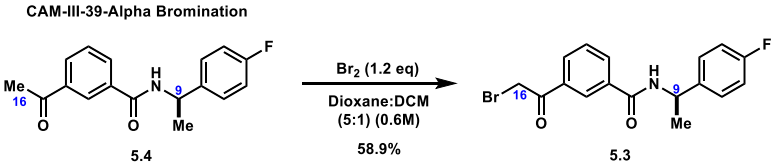


Figure 22.HRMS (-ESI) of compound 5.4: calculated C17H16FNO2 -H+ [M - H+]: 284.1092 m/z, observed 284.1089 m/z, Δ = 1.06 ppm



Reactants			*Products*	
Formula	$C_{17}H_{16}FNO_2$	Br_2	Formula	$C_{17}H_{15}BrFNO_2$
MW	285.32	159.81	MW	364.21
Limiting?	***Yes***	No	Equivalents	
Equivalents	***1.00***	***1.10***	%Completion	
Sample Mass	***1.74g***	1.07g	Expected Mass	2.22g
%Weight			Expected Moles	6.10mmol
Molarity	***500.00mM***		Measured Mass	***1.53g***
Density			Purity	
Volume	12.20mL		Product Mass	1.53g
Reactant Moles	6.10mmol	6.71mmol	Product Moles	4.20mmol
Reactant Mass	1.74g	1.07g	%Yield	68.88%

Alpha bromination to (R)-3-(2-bromoacetyl)-N-(1-(4-fluorophenyl)ethyl)benzamide (**5.3)** : To a 50 mL conical flask equipped with stir vane was added solid amide **5.4** (1.74 g, 6.10 mmol). The flask was capped with septa then thrice purged with house nitrogen and vacuum. Freshly distilled DCM (2.0 mL) added to dissolve, then solution was diluted with dioxane (10mL). Reaction flask was cooled to 0°C. Bromine (343.6 µL, 6.71 mmol) was added fast dropwise and reaction proceeded overnight. Reaction was difficult to monitor via TLC due to the polarity of compound **5.3** and **5.4** being so similar. TLC with 7.5% methanol in DCM mobile phase show compounds **5.3** and **5.4** of 0.5 Rf, UV active, and imaged in CAM stain. Product **5.3** stains slightly pinker than starting material **5.4**. Reaction condensed *in vacuo* to afford an orange/yellow crude semisolid. Crude material dissolved in hot DCM and product crashed out upon cooling. The white precipitate was then filtered to afford 1.53g (68.9% yield) of bromo amide **5.3** which was confirmed via ^{1}HNMR (**Figure 23**), ^{13}CNMR (**Figure 24**), HSQC (**Figure 25**), and HRMS (**Figure 26**). Crude material pushed forward without further purification.

^{1}H NMR (500 MHz, Chloroform-*d*) δ 1.61 (d, *J* = 7.02 Hz, 3H, 10), 4.46 (s, 2H, 16), 5.31 (p, *J* = 7.08 Hz, 1H, 9), 6.45 (d, *J* = 7.40 Hz, 1H, 8), 7.04 (td, *J* = 8.67, 2.04 Hz, 2H, 13, 13'), 7.36 (dd, *J* = 8.62, 5.37 Hz, 2H, 12, 12'), 7.50 – 7.66 (m, 1H, 2), 8.04 (dd, *J* = 8.27, 1.23 Hz, 1H, 1), 8.09 (ddd, *J* = 7.82, 1.80, 1.13 Hz, 1H, 3), 8.34 (t, *J* = 1.82 Hz, 1H, 5); HRMS (-ESI): calculated $C_{17}H_{16}BrFNO_2$ -H+ [M - H+]: 365.02497 m/z, observed 362.0196 m/z, Δ = 0.28ppm

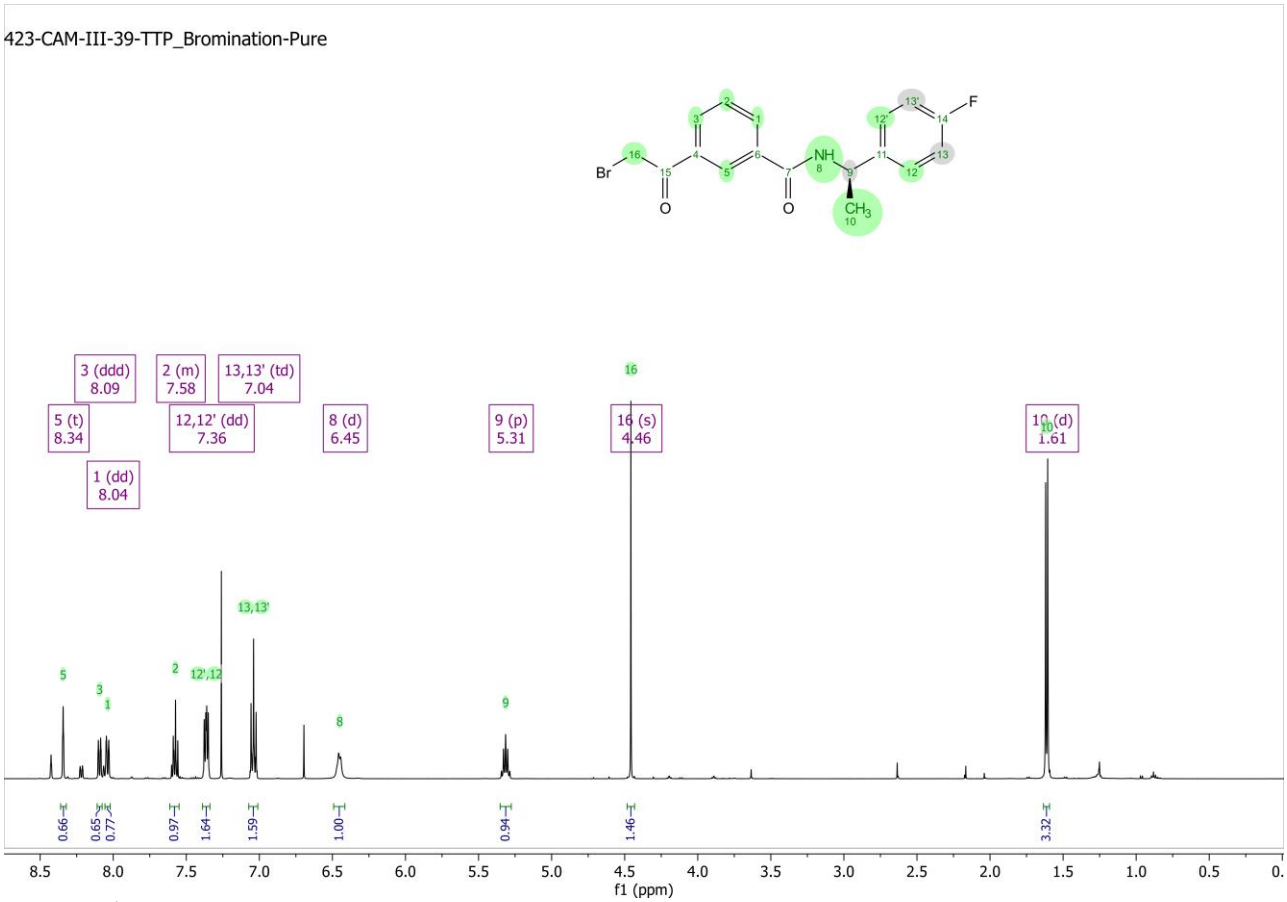


Figure 23. [1]HNMR of alpha mono brominated amide 5.3in d-chloroform

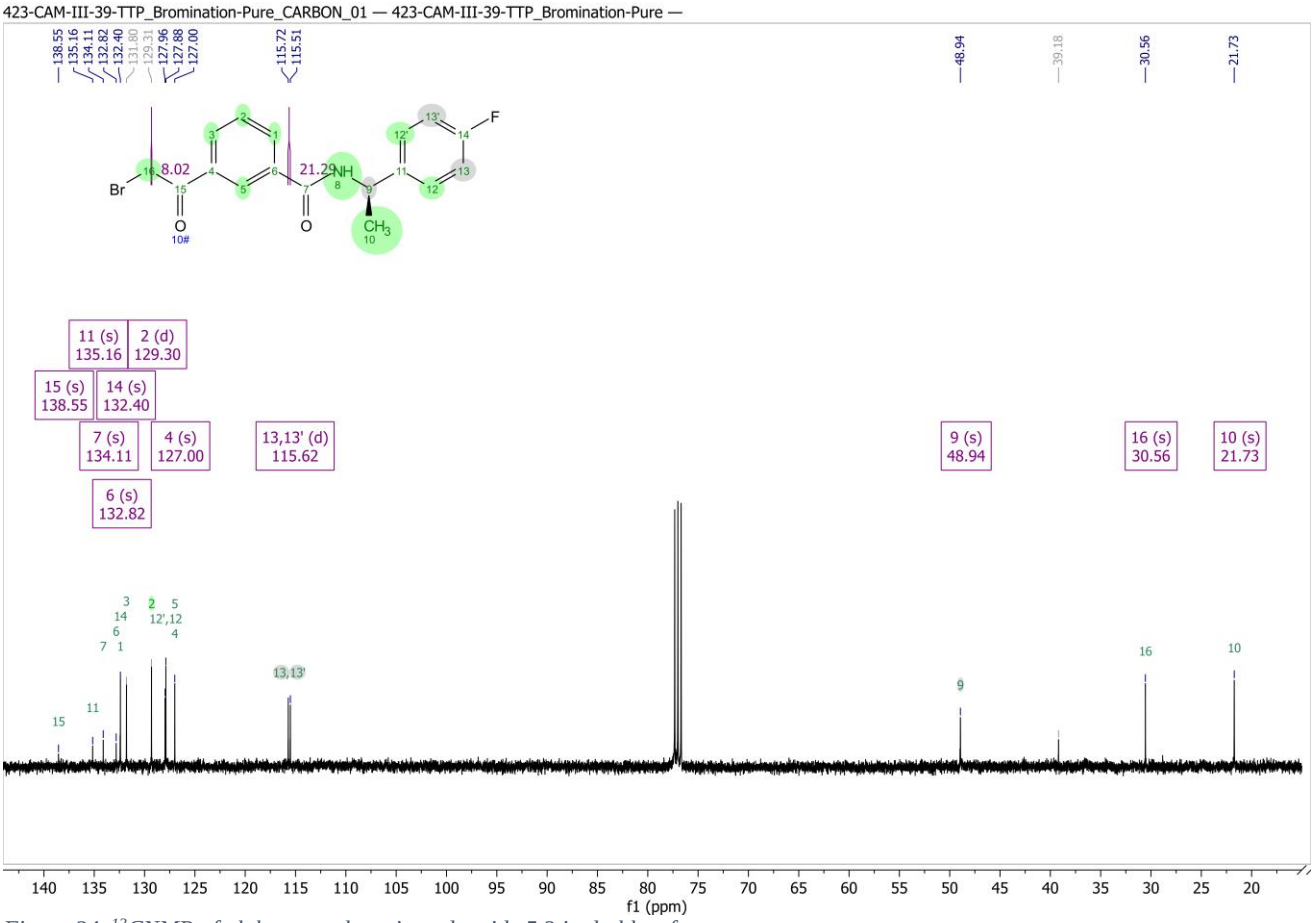


Figure 24. [13]CNMR of alpha mono brominated amide 5.3 in d-chloroform

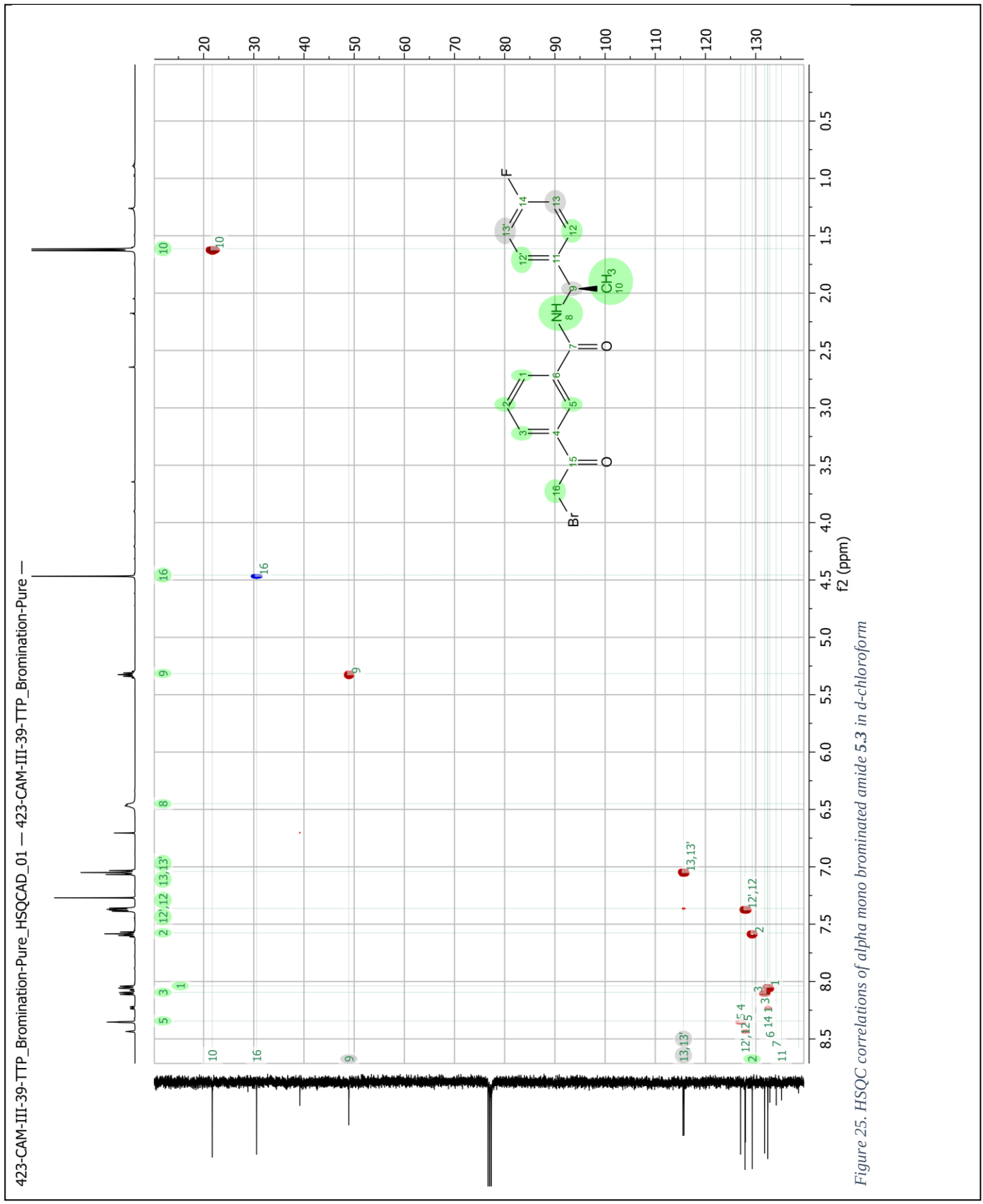


*Figure 25. HSQC correlations of alpha mono brominated amide **5.3** in d-chloroform*

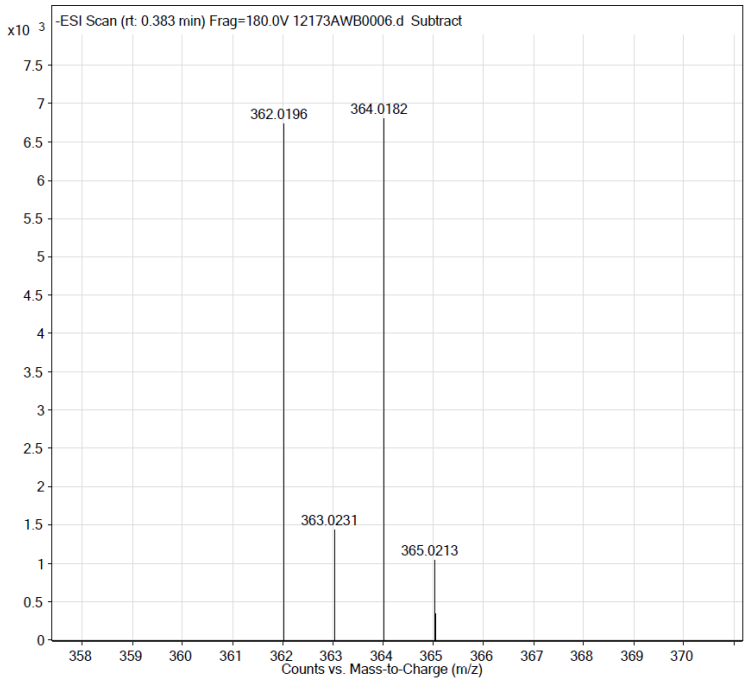
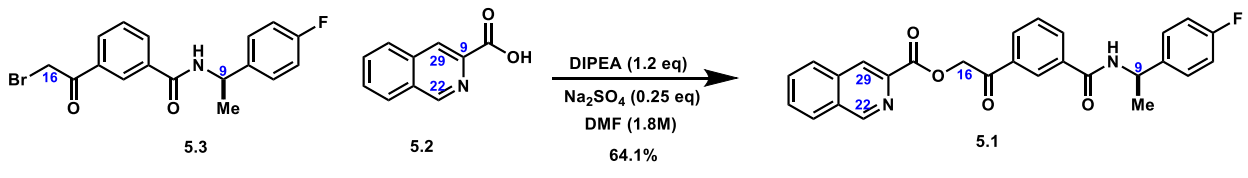


Figure 26. HRMS (-ESI) of compound 5.3: calculated C17H16BrFNO2 -H+ [M - H+]: 365.02497 m/z, observed 362.0196 m/z, Δ = 0.276228ppm

CAM-III-57-TTP Isoquinoline Coupling



Reactants						*Products*	
Formula	$C_{17}H_{15}BrFNO_2$	$C_{10}H_7NO_2$	$C_8H_{19}N$	Na_2O_4S	C_3H_7NO	Formula	$C_{27}H_{21}FN_2O_4$
MW	364.21	173.17	129.25	142.04	73.10	MW	456.47
Limiting?	***Yes***	No	No	No	No	Equivalents	
Equivalents	***1.00***	***1.01***	***1.20***	***0.25***		%Completion	
Sample Mass	***137.00mg***	65.79mg	58.34mg	13.36mg	27.49mg	Expected Mass	171.70mg
%Weight						Expected Moles	376.15µmol
Molarity		***1.80M***				Measured Mass	***117.60mg***
Density			***742.00mg/mL***			Purity	
Volume		211.06mL	78.63mL			Product Mass	117.60mg
Reactant Moles	376.15µmol	379.91µmol	451.38µmol	94.04µmol	376.15µmol	Product Moles	257.63µmol
Reactant Mass	137.00mg	65.79mg	58.34mg	13.36mg	27.49mg	%Yield	68.49%

Base mediated coupling to (R)-2-(3-((1-(4-fluorophenyl)ethyl)carbamoyl)phenyl)-2-oxoethyl isoquinoline-3-carboxylate (**5.1**): Isoquinoline-1-carboxylic acid (**5.2,** 211.06 µl, 379.91 µmol) and sodium sulfate (13.36 mg, 94.04 µmol) were weighed out into 25 ml pear-shaped flask. A large spin vane was added, flask was septa sealed, then thrice purged with house nitrogen and vacuum. DMF (1.0 mL) added to flask followed by DIPEA (78.63 µL) to dissolve solids. Reaction was cooled to 0°C. Starting material bromo amide, **5.3**, was dissolved in DMF (1.0 mL) then added to reaction flask via syringe slow drop-wise. Reaction proceeded over 90 minutes and was monitored via TLC with 50% acetone in hexanes as mobile phase. Starting material **5.3** observed at 0.7Rf, product UV active and imaged in CAM stain. Reaction stopped progressing after 60 minutes and remaining 20% starting material by TLC could not be pushed further. Quenched with distilled water and solution turned translucent with white precipitate. Precipitate filtered and washed with cold distilled water then dissolved in ethyl acetate. The organic solution was washed twice with saturated sodium bicarbonate then brine. Back extracted with ethyl acetate twice. Dried over sodium sulfate, filtered through cotton plug, and then condensed down *in vacuo*. Crude material flashed through 40 mL silica with 30% acetone in hexanes as mobile phase to afford 117.6mg (68.49% yield) of white solid isoquinoline amide **5.1**. Structure confirmed via ^{1}HNMR (**Figure 28**) and HRMS (**Figure 27**).

^{1}H NMR (500 MHz, Chloroform-*d*) δ 1.63 (d, *J* = 6.92 Hz, 3H, 10), 5.33 (p, *J* = 7.08 Hz, 1H, 9), 5.69 (d, *J* = 0.95 Hz, 2H, 16), 6.38 (d, *J* = 7.78 Hz, 1H, 8), 7.05 (t, *J* = 8.64 Hz, 2H, 13, 13'), 7.37 (t, *J* = 6.67 Hz, 2H, 12, 12'), 7.62 (t, *J* = 7.75 Hz, 1H, 5), 7.65 (ddd, *J* = 8.12, 6.91, 1.18 Hz, 1H, 2), 7.87 (ddd, *J* = 8.45, 6.92, 1.44 Hz, 1H, 26), 7.97 (d, *J* = 8.11 Hz, 1H, 27), 8.07 (dd, *J* = 8.94, 1.36 Hz, 1H, 1), 8.11 (dt, *J* = 7.76, 1.35 Hz, 1H, 3), 8.20 (dd, *J* = 8.56, 1.09 Hz, 1H, 24), 8.37 (t, *J* = 1.71 Hz, 1H, 5), 8.97 (d, *J* = 1.96 Hz, 1H, 29), 9.53 (d, *J* = 2.14 Hz, 1H, 22). HRMS (-ESI): calculated $C_{27}H_{21}FN_2O_4$-H+ [M - H+]: 455.1413 m/z, observed 455.1417 m/z, Δ = 0.88 ppm

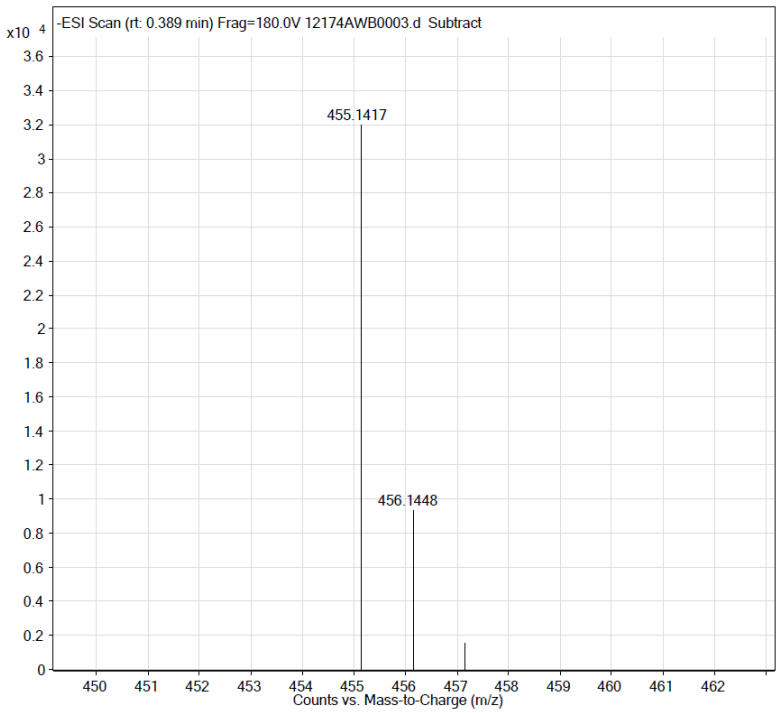


Figure 27. HRMS (-ESI) of compound 5.1: calculated C27H21FN2O4-H+ [M - H+]: 455.1413 m/z, observed 455.1417 m/z, Δ = 0.878848 ppm

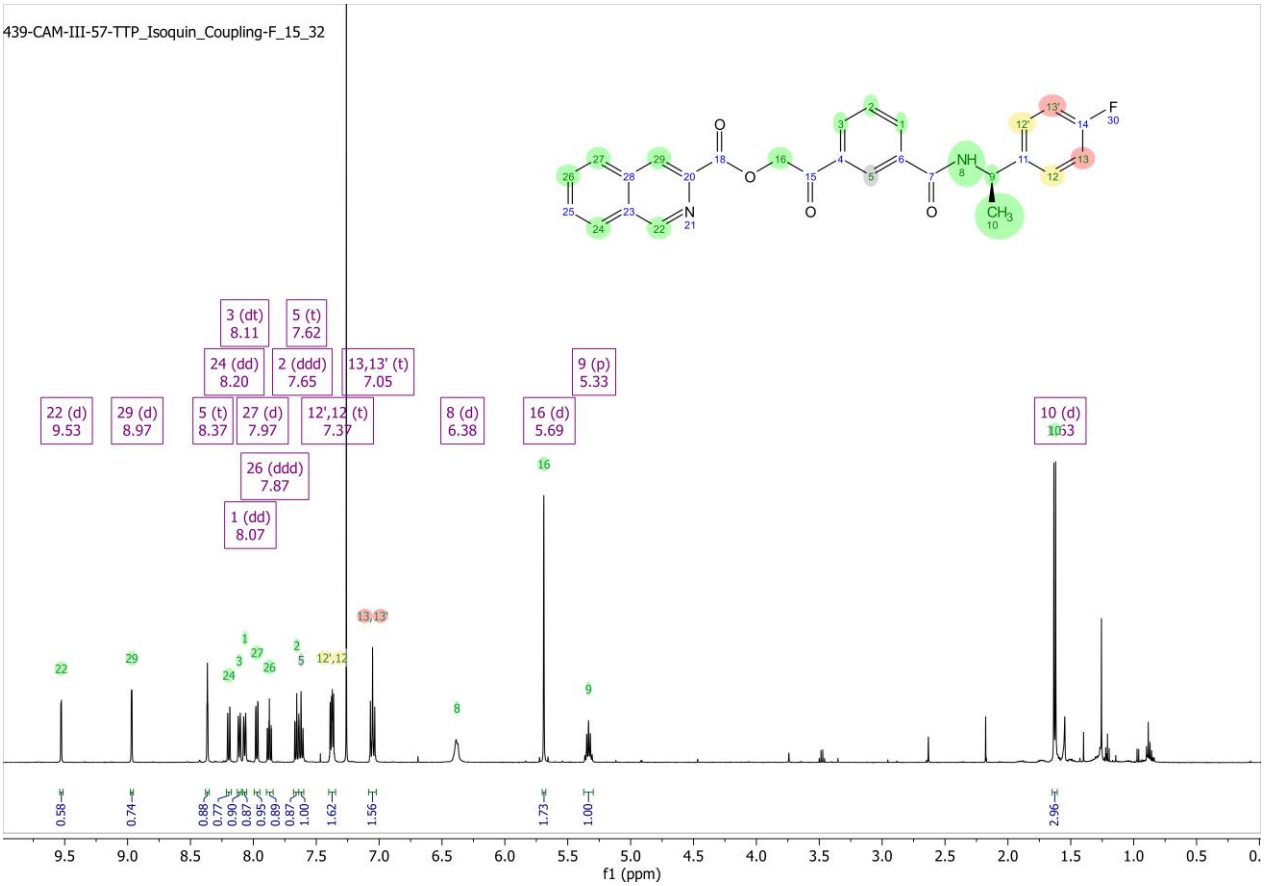
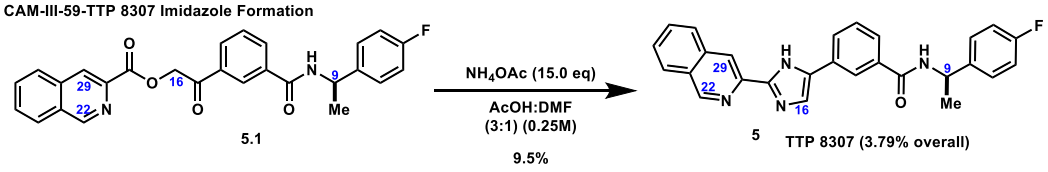


*Figure 28. [1]HNMR of Isoquinoline Amide **5.1** in d-chloroform*



Reactants					*Products*	
Formula	$C_{27}H_{21}FN_2O_4$	$C_2H_7NO_2$	C_3H_7NO	$C_2H_4O_2$	Formula	$C_{27}H_{21}FN_4O$
MW	456.47	77.08		60.05	MW	436.49
Limiting?	***Yes***	No	No	No	Equivalents	
Equivalents	***1.00***	***15.00***			%Completion	
Sample Mass	***110.00mg***	278.63mg		14.47mg	Expected Mass	105.18mg
%Weight					Expected Moles	240.98µmol
Molarity	***250.00mM***				Measured Mass	***10.00mg***
Density					Purity	
Volume	963.91mmL				Product Mass	10.00mg
Reactant Moles	240.98µmol	3.61mmol	240.98µmol	240.98µmol	Product Moles	22.91µmol
Reactant Mass	110.00mg	278.63mg		14.47mg	%Yield	9.51%

<u>Imidazole formation to TTP-8307 (**5**):</u> Starting material **5.1** (110 mg, 240.98 µmol) dissolved in DMF (0.5 mL) and acetic acid (1.4 mL) then placed into 25 mL round bottom flask equipped with spin vane. Solid ammonium acetate (278.6 mg, 3.61 mmol) was added to flask. A water jacketed condenser attached to flask and bulk refluxed to 150°C for 90 minutes. Reaction turned yellow upon heating. Reaction monitored via TLC with 50% acetone in hexanes mobile phase. Starting

material **5.1** observed at 0.6 Rf. Product observed at 0.2 Rf was UV active and imaged in CAM stain. Upon complete conversion via TLC, reaction was cooled to room temp then quenched with cold distilled water, solution turned cloudy. Bulk washed with saturated sodium bicarbonate twice then aqueous phase back extracted with ethyl acetate. Organic phases combined then shaken in 4N $HCl_{(aq)}$ and white/yellow precipitate crashed out. The organic and aqueous layers were filtered through cotton plug. The precipitate was then dissolved in methanol and condensed down *in vacuo* to produce a white/yellow solid. Crude mixture purified via HPLC-MS using Shimadzu UFLC instrumentation equipped with Luna 5 μm C8(2) 100Å column and isocratic 80% methanol to 0.1% formic acid/water. 10.0 mg of pure TTP 8307 was obtained after HPLC purification (9.5% yield). TTP 8307 structure was fully characterized as shown in **Table 4**, ^{1}HNMR (**Figure 29**), ^{13}CNMR (**Figure 30**), HRMS (**Figure 31 & 32**) COSY (**Figure 33 & 34**), and HSQC (**Figure 35 & 36**). Biological purity determined via chromatogram and is 95.8% pure at 254 nm absorbance and 98.1% pure at 280 nm absorbance, as shown in **Figure 37** and **Table 5**.

^{1}H NMR (600 MHz, Methanol-d_4) δ 1.59 (d, J = 7.05 Hz, 3H, 10), 5.27 (q, J = 7.16 Hz, 1H, 9), 7.01 – 7.13 (m, 2H, 13, 13'), 7.41 – 7.52 (m, 2H, 12, 12'), 7.68 (t, J = 7.80 Hz, 1H, 2), 7.82 (ddd, J = 8.17, 6.92, 1.13 Hz, 1H, 26), 7.96 (dt, J = 8.19, 1.23 Hz, 1H, 1), 8.00 (ddd, J = 8.42, 6.88, 1.41 Hz, 1H, 25), 8.04 (ddd, J = 7.77, 1.89, 1.00 Hz, 1H, 3), 8.14 – 8.23 (m, 3H, 16, 24, 27), 8.33 (t, J = 1.76 Hz, 1H, 5), 9.04 (d, J = 2.28 Hz, 1H, 29), 9.43 (d, J = 2.34 Hz, 1H, 22).

HRMS (+ESI): calculated $C_{27}H_{21}FN_4O$ [M + H+]: 437.1772 m/z, observed 437.1761 m/z, Δ = 2.5 ppm

*Table 4. HNMR, CNMR, COSY, & HSQC spectrometric characterization of TTP-8307 (5).*HSQC correlation not observed for C16-H16 because of the coupling constant of imidazole being outside of the NMR acquisition parameters.*[9]

Atom	Chemical Shift	COSY	HSQC	Atom	Chemical Shift	COSY	HSQC
1 C	127.92		1	13' C	114.70		13'
H	7.96	2, 5	1	H	7.07	12, 12'	13'
2 C	129.32		2	14 C			
H	7.68	1, 3	2	15 C			
3 C	128.43		3	16 C	117.22		*
H	8.04	2	3	H	8.17		*
4 C				18 C			
5 C	124.88		5	19 N			
H	8.33	1	5	H			
6 C				20 C			
7 C				22 C	146.19		22
8 N				H	9.43	29	22
H				23 C			
9 C	49.03		9	24 C	127.85		24
H	5.27	10	9	H	8.17	25, 26	24
10 C	20.81		10	25 C	133.19		25
H3	1.60	9	10	H	8.00	24, 26, 27	25
11 C				26 C	128.64		26
12 C	127.70		12	H	7.82	24, 25, 27	26
H	7.44	13, 13'	12	27 C	128.86		27
12' C	127.70		12'	H	8.17	25, 26	27
H	7.44	13, 13'	12'	28 C			
13 C	114.70		13	29 C	137.68		29
H	7.07	12, 12'	13	H	9.04	22	29

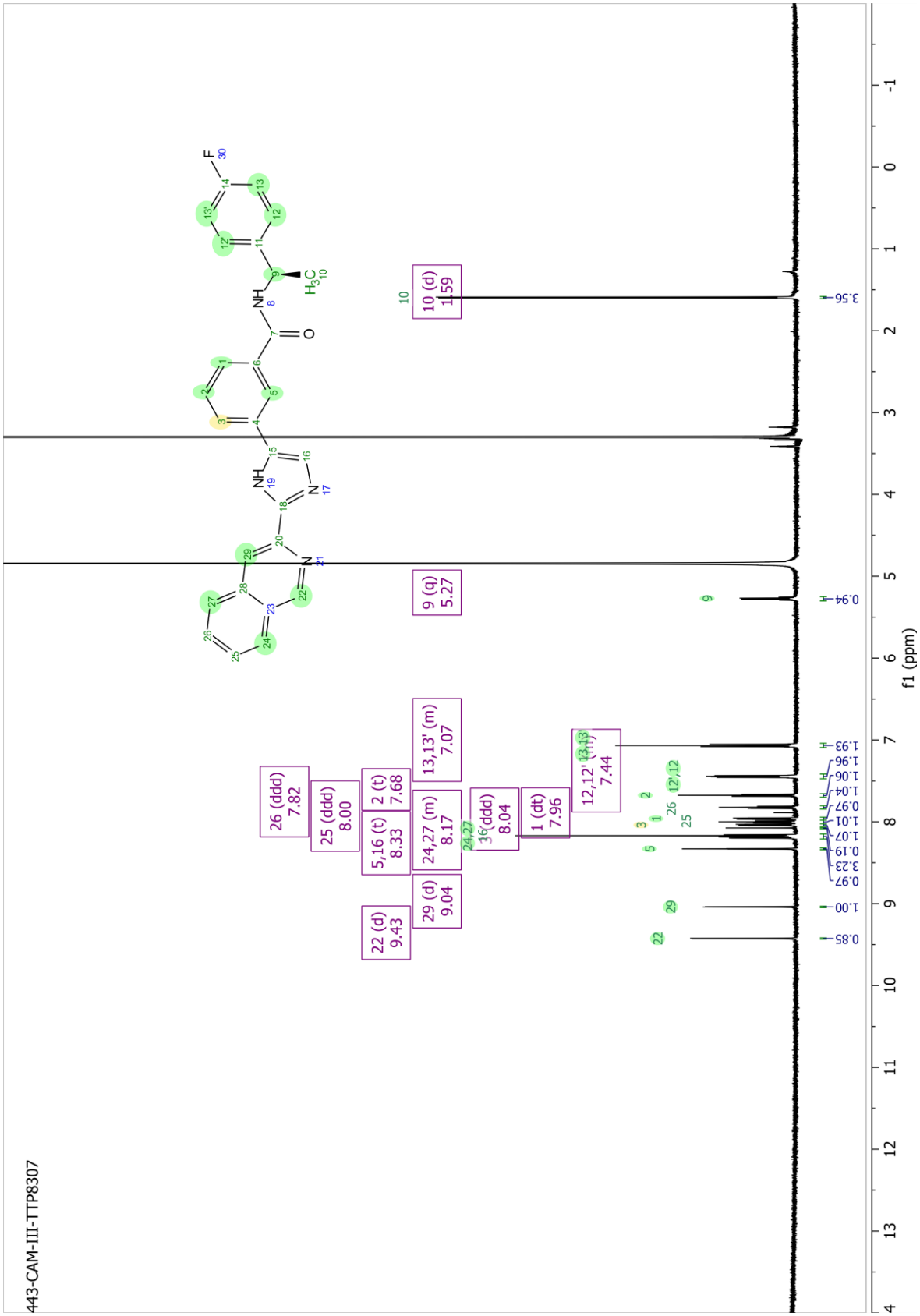


Figure 29. ^{1}HNMR of TTP (5) in d-Methanol

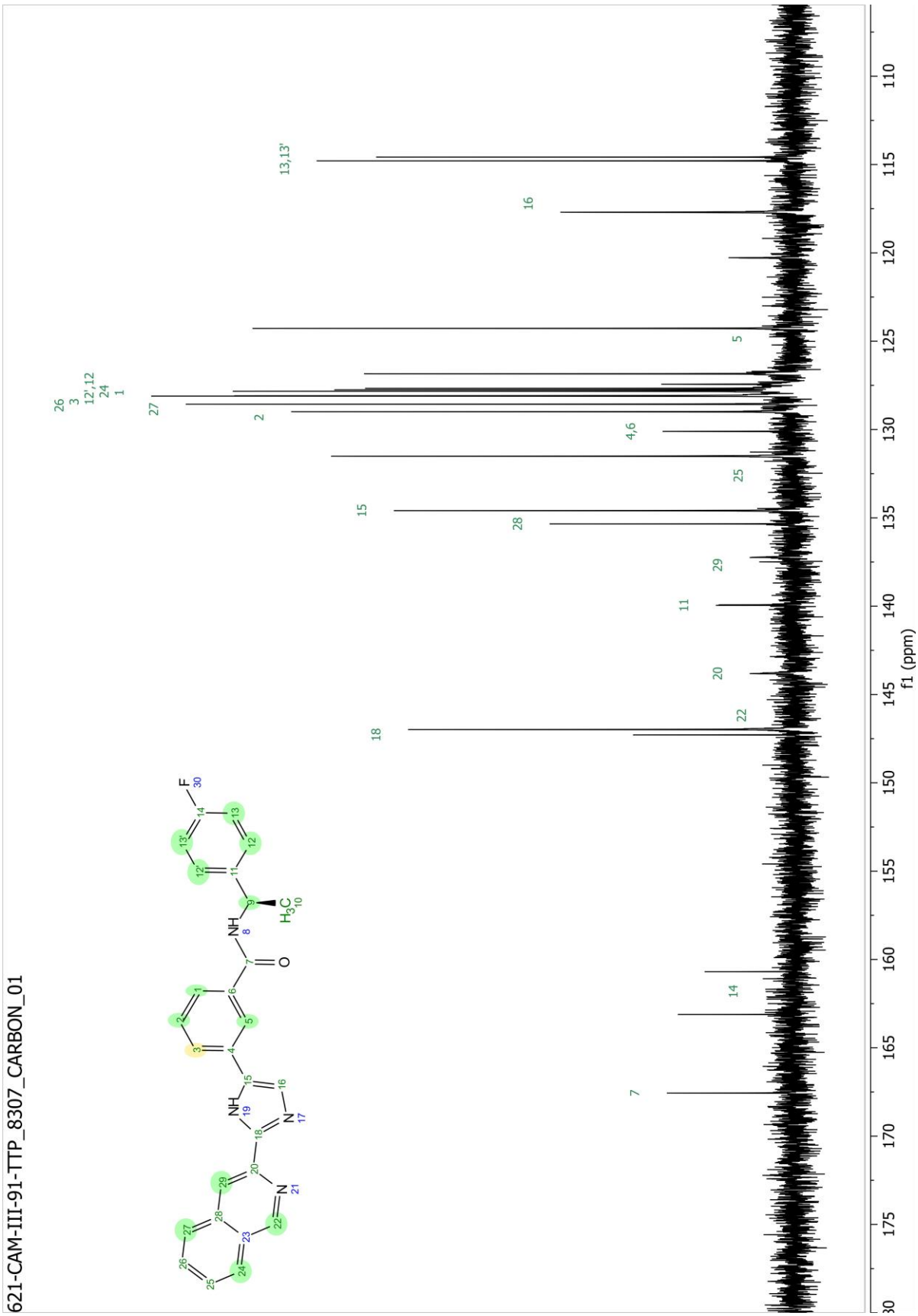


Figure 30. ^{13}CNMR of TTP (5) in d-Methanol

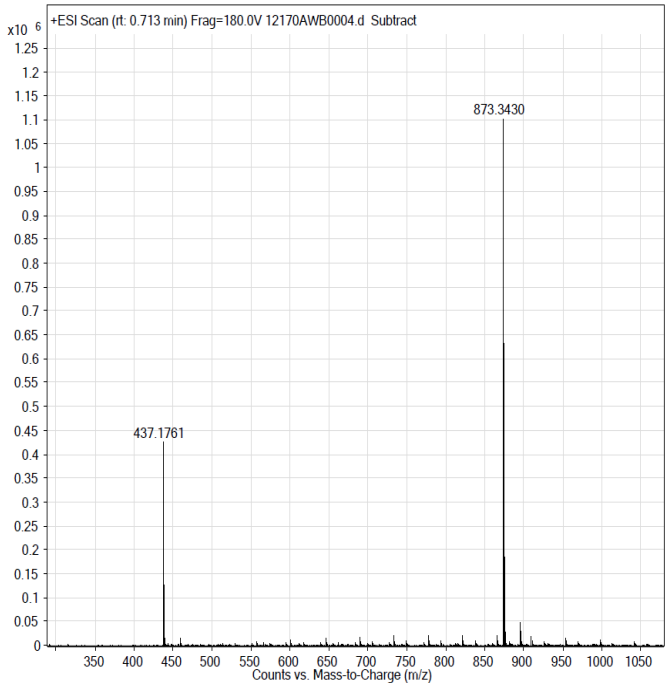


Figure 31. HRMS (+ESI) of TTP-8307 (5): calculated C27H21FN4O [M + H+]: 437.1772 m/z, observed 437.1761 m/z, Δ = 2.52 ppm

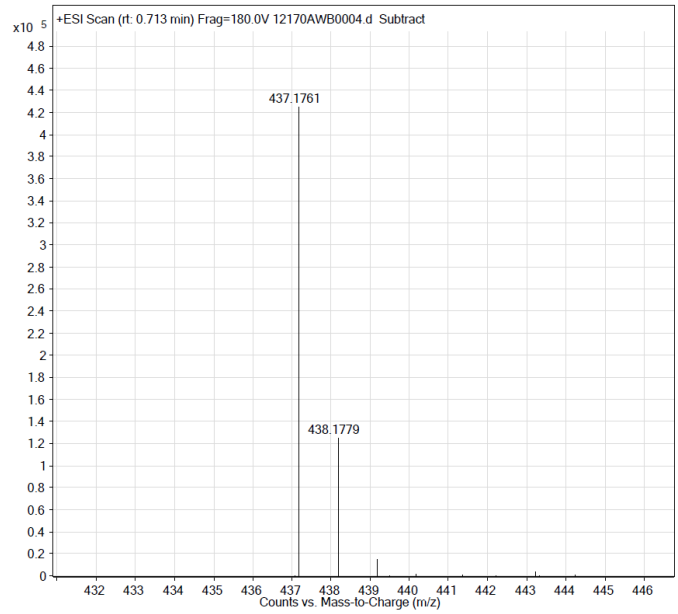


Figure 32. Magnified HRMS (+ESI) of TTP-8307 (5): calculated C27H21FN4O [M + H+]: 437.1772 m/z, observed 437.1761 m/z, Δ = 2.52 ppm

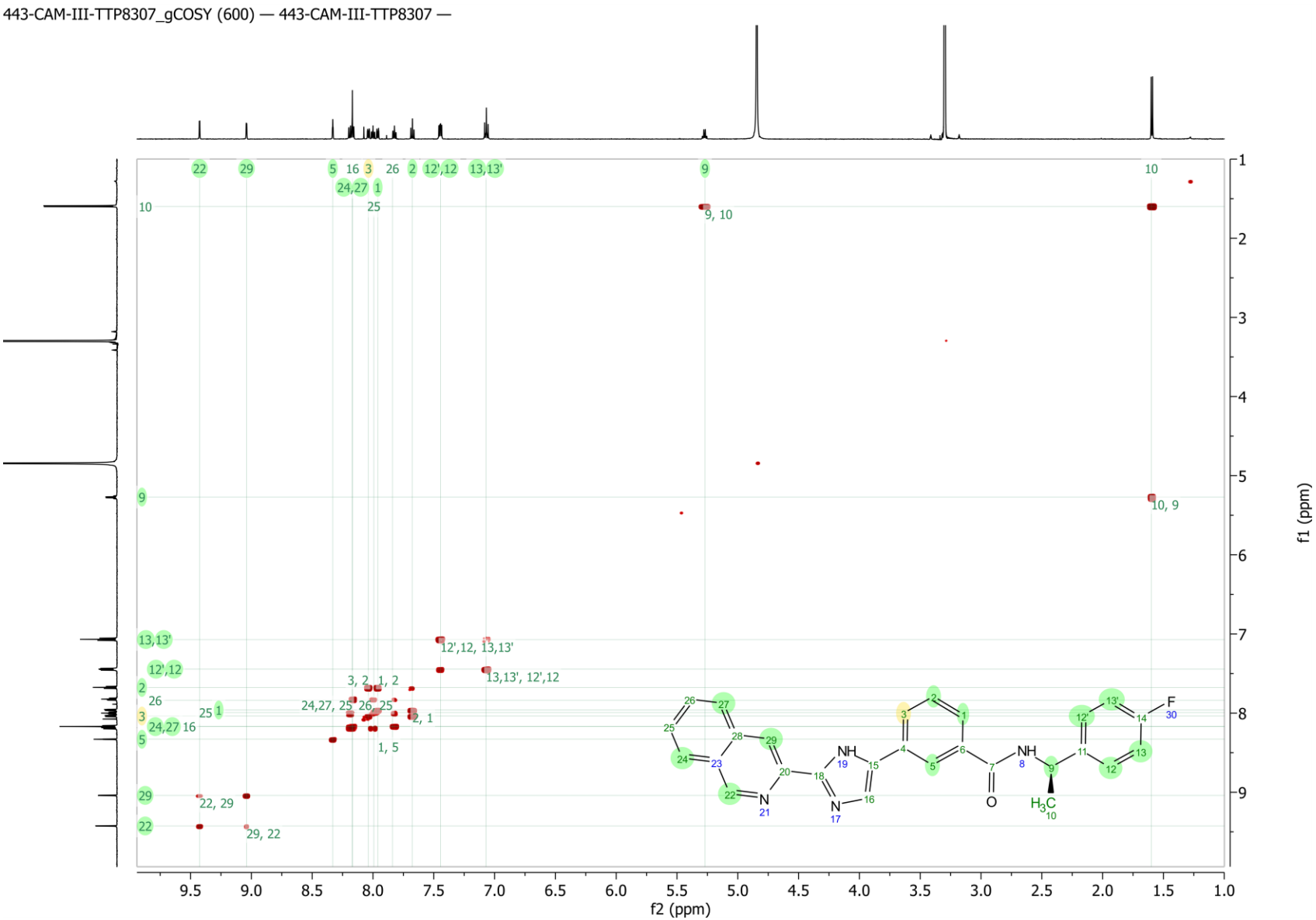


Figure 33. COSY correlation of TTP (5) in d-Methanol.

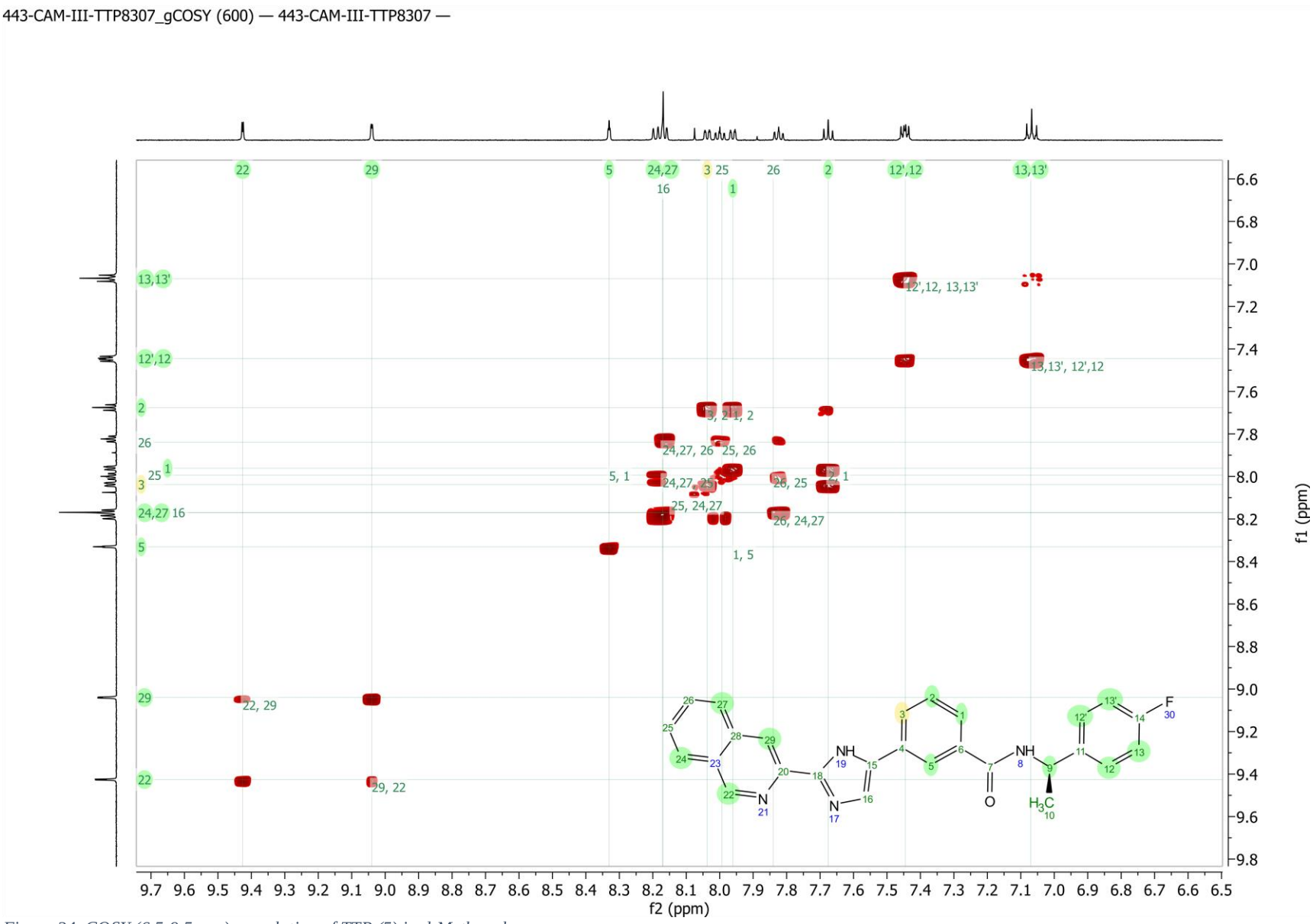


Figure 34. COSY (6.5-9.5ppm) correlation of TTP (5) in d-Methanol.

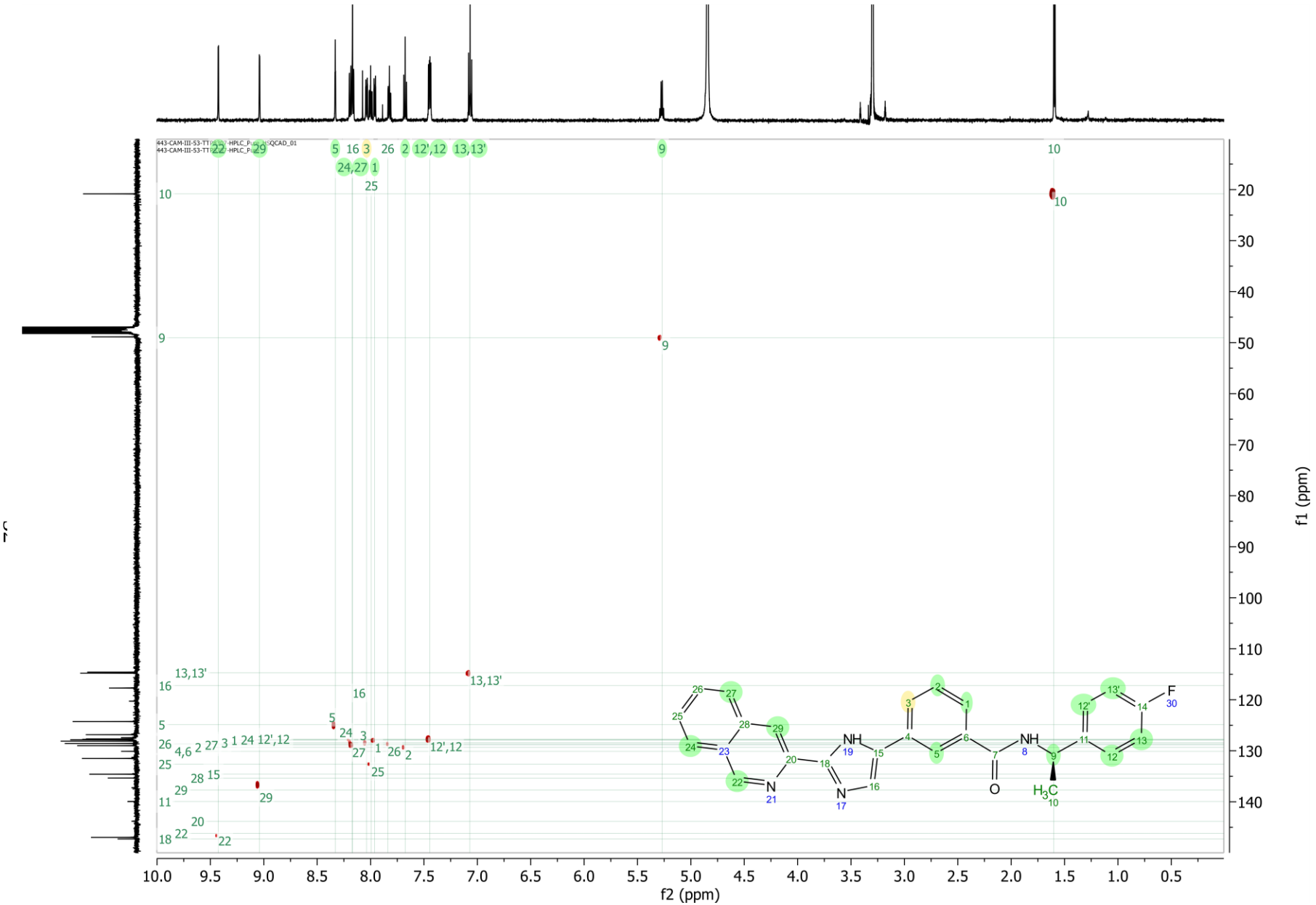


Figure 35. HSQC correlation of TTP (5) in d-Methanol.

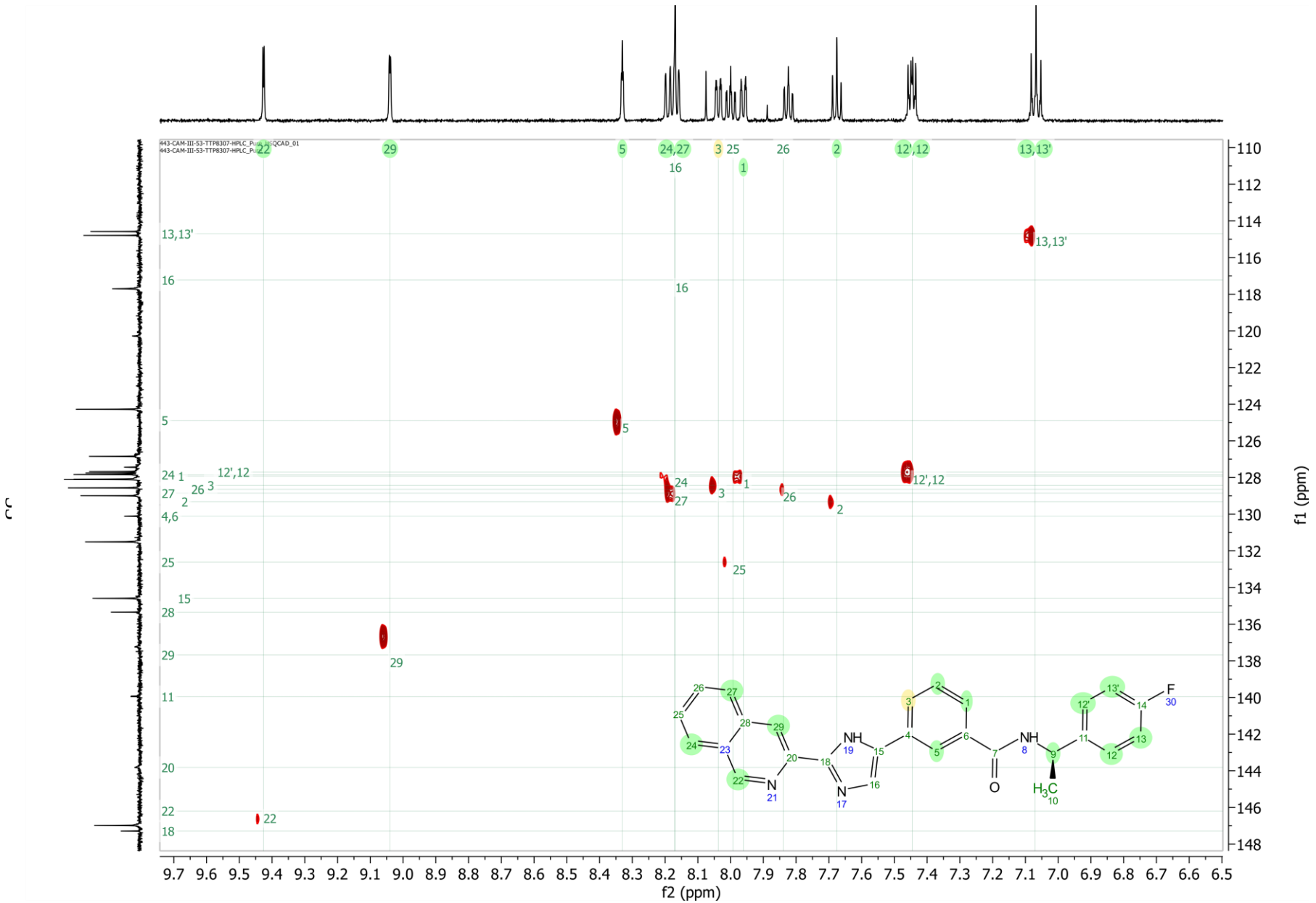


Figure 36. HSQC (6.5-9.6 ppm) of TTP (5) in d-Methanol.

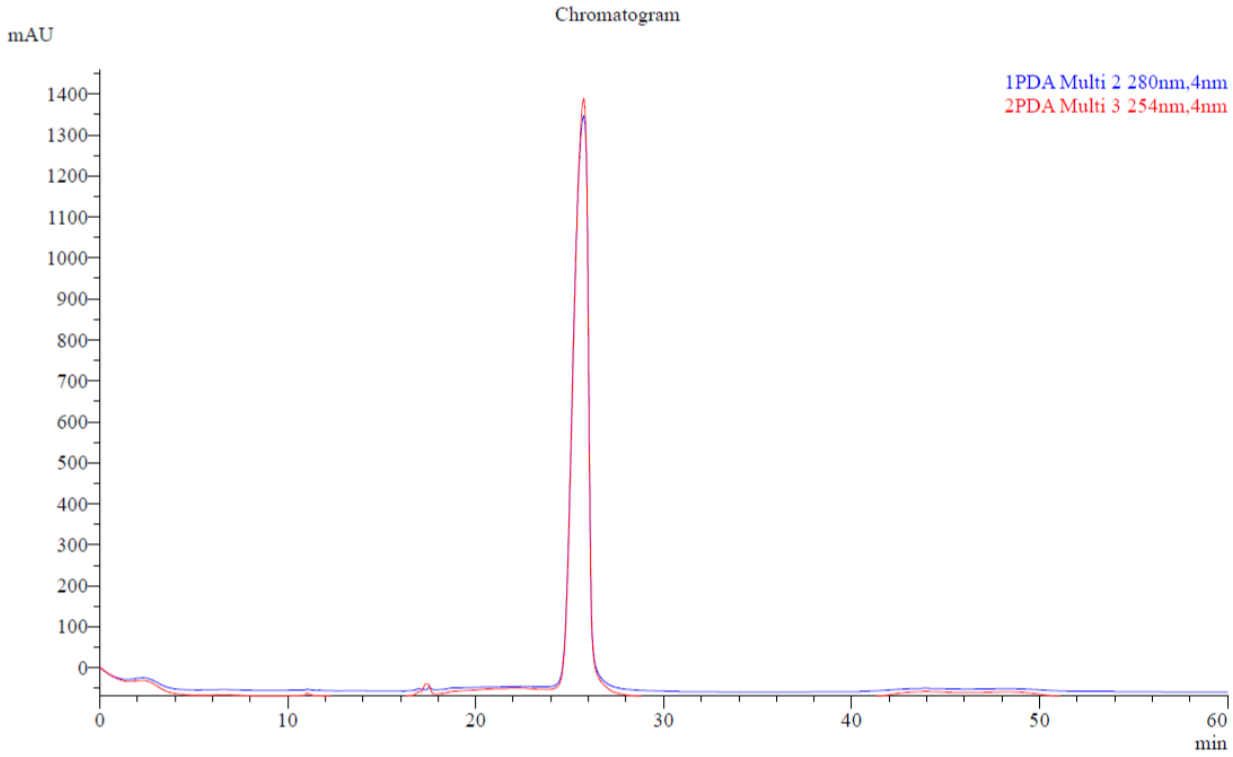


Figure 37. Multi-Chromatogram analysis of TTP-8307 (5) at max absorbance 280nm (shown in blue) and 254nm (shown in red).

Table 5. Tabulated multi-chromatogram analysis of TTP 8307 (5). At 280 nm, concentration of TTP-8307 is 98.3%. At 254 nm, concentration of TTP-8307 is 95.8%.

Peak Table

FRC Signal

Peak#	Ret. Time	Area	Height	Conc.
Total				

PDA Ch2 280nm

Peak#	Ret. Time	Area	Height	Conc.
1	2.288	877946	11408	1.094
2	6.293	1652	59	0.002
3	11.027	42452	2982	0.053
4	11.673	7005	499	0.009
5	13.301	5679	285	0.007
6	16.982	114538	5023	0.143
7	17.228	29067	2978	0.036
8	17.536	92803	7931	0.116
9	18.783	77174	2402	0.096
10	20.597	109585	381	0.137
11	21.931	12073	325	0.015
12	22.773	31134	816	0.039
13	25.748	78831210	1397048	98.254
Total		80232317	1432136	100.000

PDA Ch3 254nm

Peak#	Ret. Time	Area	Height	Conc.
1	2.240	823032	10530	0.991
2	11.031	142094	8004	0.171
3	11.160	82195	5614	0.099
4	11.670	54131	2124	0.065
5	13.292	4315	205	0.005
6	17.000	210389	9872	0.253
7	17.383	690872	27340	0.832
8	21.280	422617	521	0.509
9	25.749	79634715	1448529	95.848
10	48.289	1019685	6040	1.227
Total		83084043	1518777	100.000

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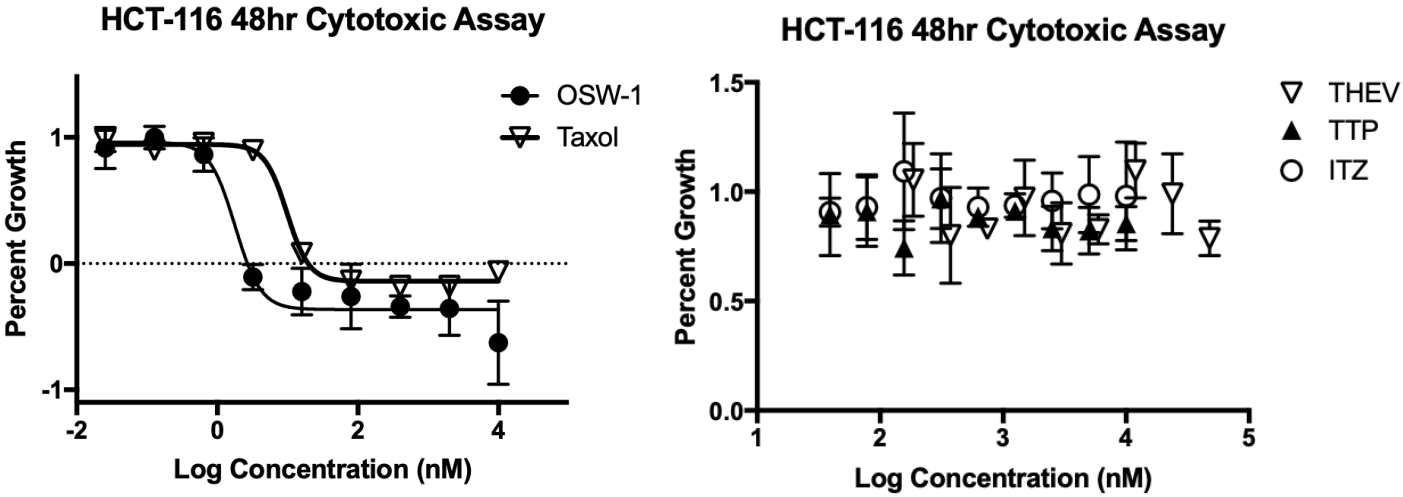
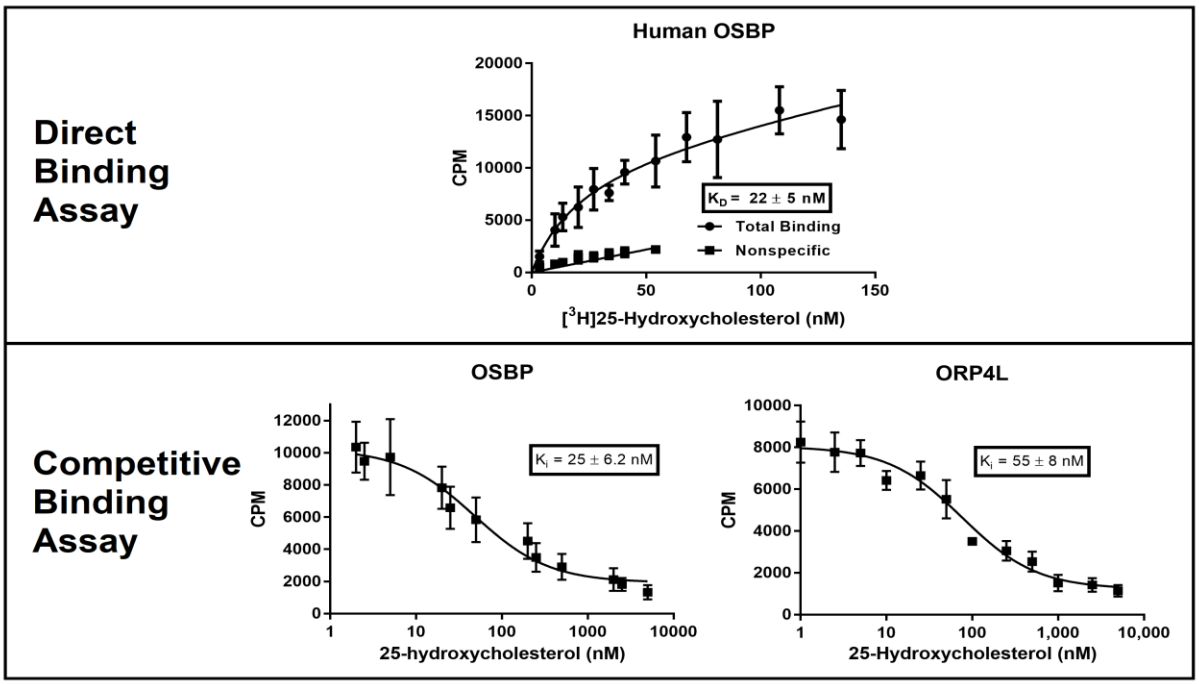
(9) Pachler, K. G. R.; Pachter, R.; Wessels, P. L. Carbon-13□proton Coupling Constants in N-substituted Imidazoles. A 13C NMR Study and MO Calculations. *Org. Magn. Reson.* **1981**, *17* (4), 278–284.

Supplemental Figures.



	Average GI_{50} (nM)				
	OSW-1	**Taxol**	**THEV**	**ITZ**	**TTP**
HCT-116	2.3 ± 1.1	9.9 ± 3.0	-	-	-

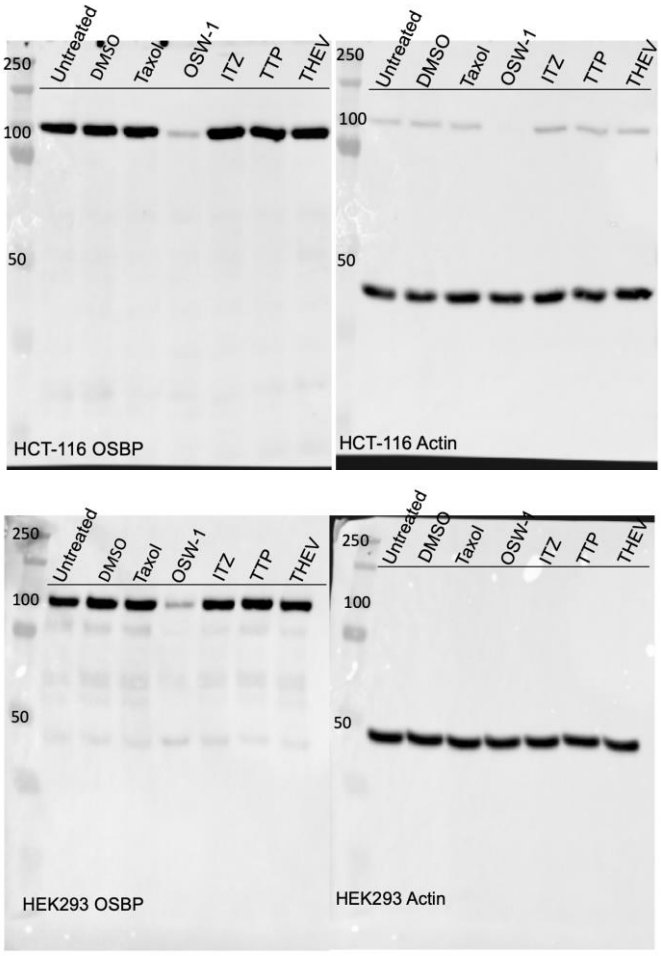
Supplemental Figure 1. Antiviral Compound Cytotoxic Assay in HCT-116.
Representative cytotoxic assay curves and average GI_{50} in HCT-116 cells for OSW-1, Taxol, THEV, TTP, and ITZ (N=3). Taxol was used as a positive control. Results show that only OSW-1 and Taxol are cytotoxic after 48hr treatment at the indicated concentrations. GI_{50} generated for OSW-1 and Taxol are consistent with previously published values by Burgett et al., 2011. Due to issues with solubility in complete cell media, in addition to the lack of cytotoxicity, TTP and ITZ were administered at the highest soluble concentration (10µM).



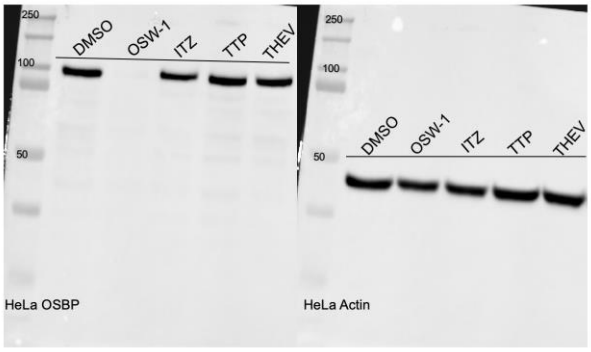
Supplemental Figure 2. 25-Hydroxycholesterol OSBP Binding Curves.
(Top) Representative binding curve for direct binding assay to establish K_D for 25-hydroxycholesterol for human OSBP used for the competitive binding assay. K_D for 25-hydroxycholesterol in ORP4L was used based off previously published values by Burgett et al., 2011. (Bottom) Representative binding curves for the competitive binding assay for 25-hydroxycholesterol in both OSBP and ORP4L.

Appendix.

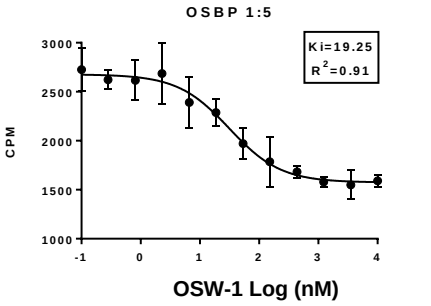
Western Blots Results from Independent Experiments for Figure 1A (Main Text)

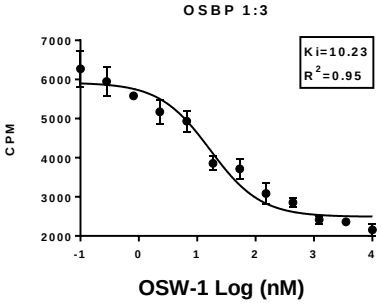


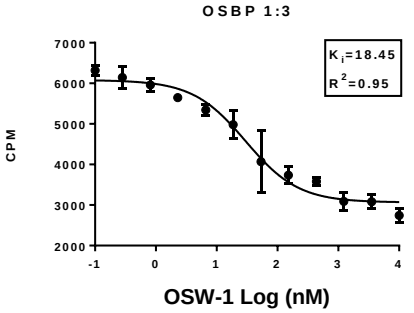
Western Blots Results from Independent Experiments for Figure 1B (Main Text)

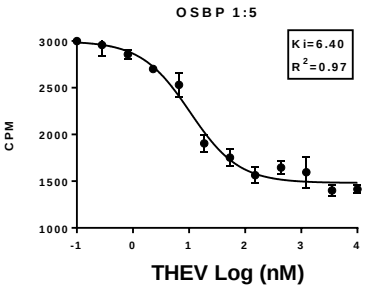


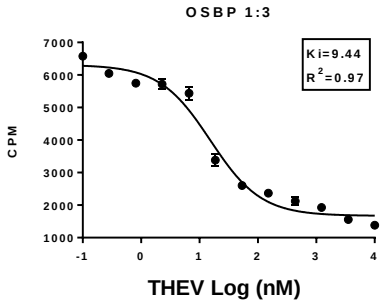
Independent Binding Experiment Results for Figure 3A (Main Text)

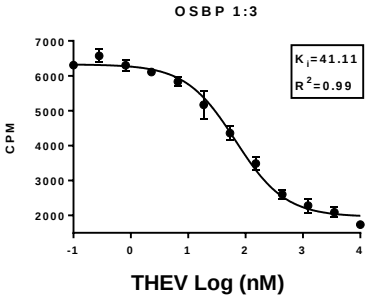


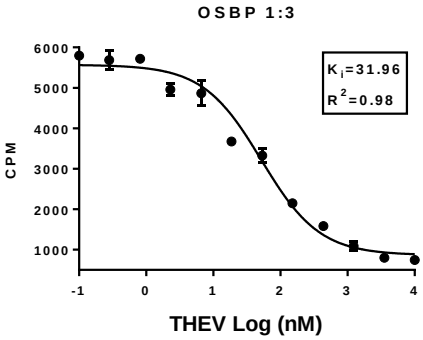


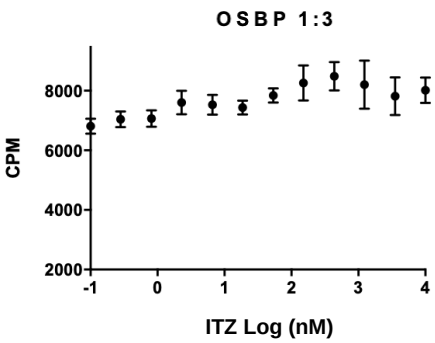


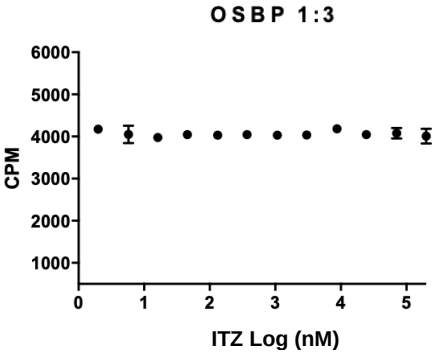


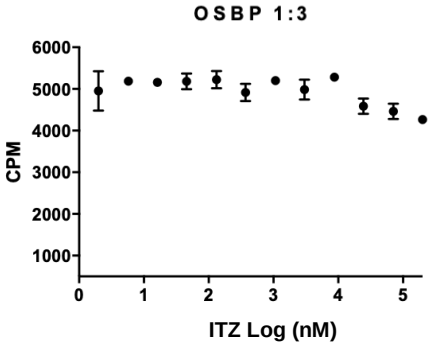


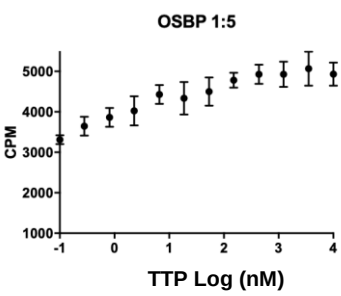


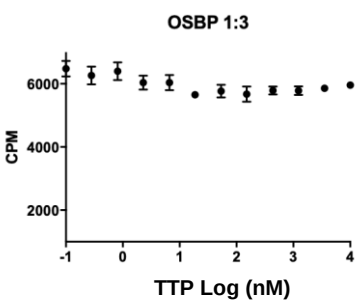


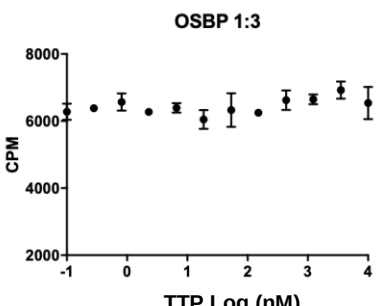


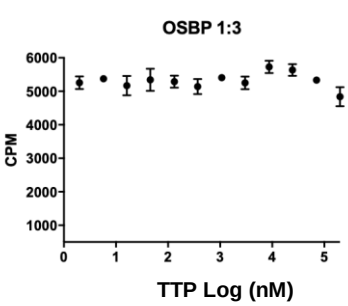




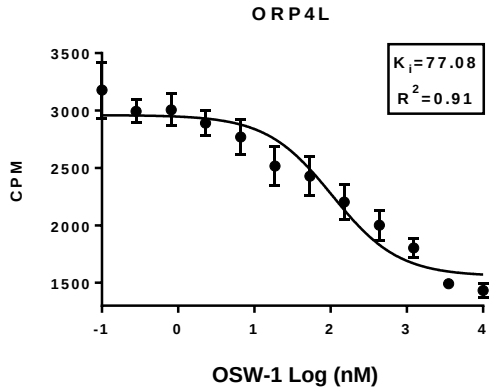


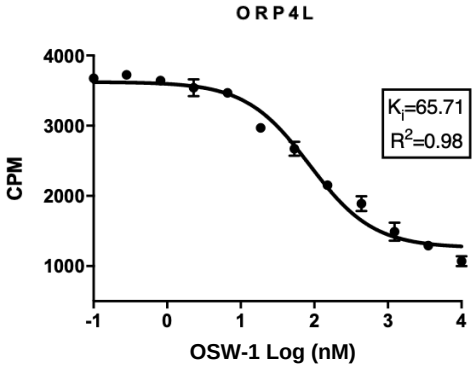


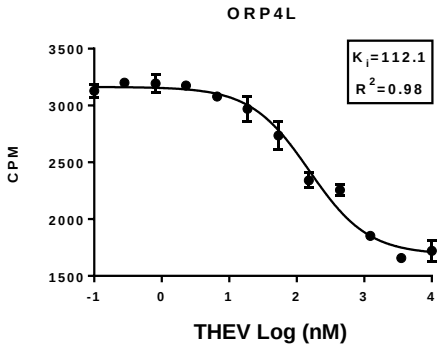


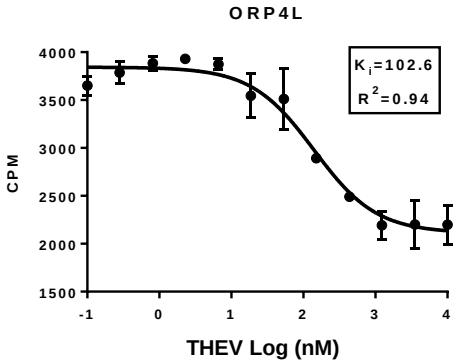


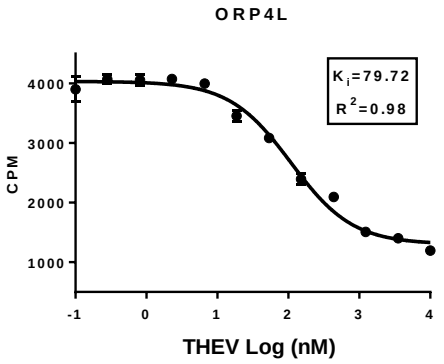
Independent Binding Experiment Results for Figure 3B (Main Text)

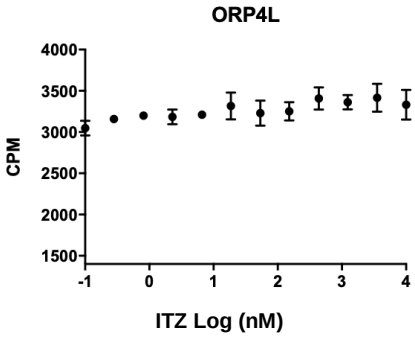


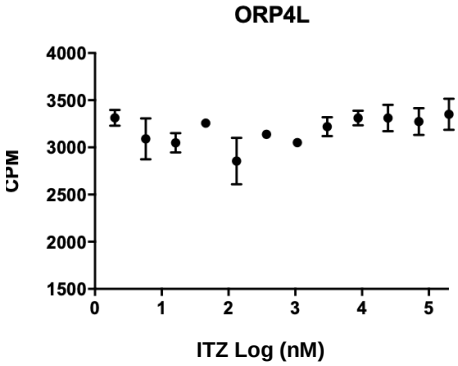


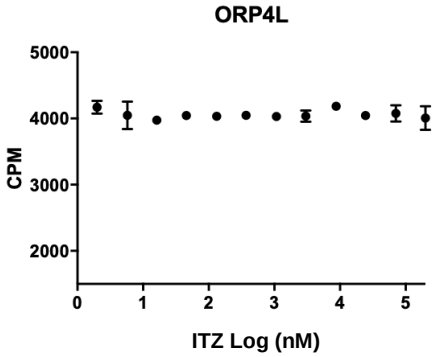


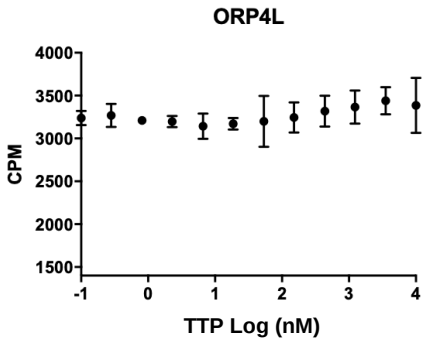


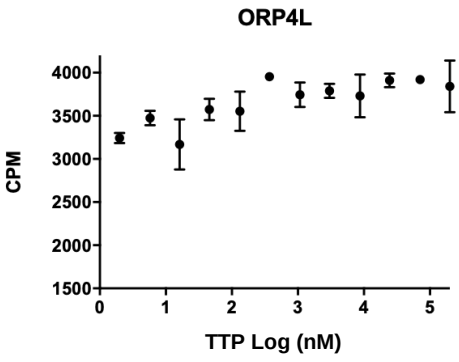


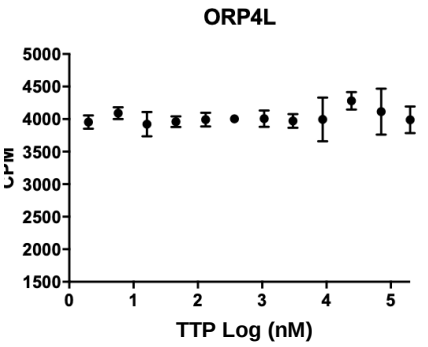












Western Blots Results from Independent Experiments for Figure 4C (Main Text)